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THE PROGNOSTIC VALUE OF AMINOPHYLLINE IN THE SELECTION OF PATIENTS FOR MITRAL VALVOTOMY

(PRELIMINARY REPORT)

M. J. DULFANO, M.D., J. YAHNI, M.D., M. TOOR, M.D., N. ROSEN, M.D., AND L. LANGER, M.D.
PETAH TIQVA, ISRAEL

CARDIAC catheterization has proved to be of some help in the assessment of indications in performing mitral valvotomy in cases of mitral stenosis. This procedure is useful in determining the degree of impairment but not in the prediction of the benefit to be derived from the operation. While in some patients valvotomy has produced striking benefits, characterized by a great diminution of pulmonary artery pressure, other patients have not shown appreciable changes. Some workers have assumed that reversible changes of a vasoconstrictive type may be present in the pulmonary circulation.[1-3] The problem arises of how to estimate preoperatively this amount of reversible changes. With this aim, we have studied, by means of cardiac catheterization, our cases of mitral stenosis, both preoperatively and postoperatively. We tried the effects of a single intravenous dose of aminophylline at the time of the preoperative cardiac catheterization and correlated the hemodynamic results with the clinical status, pulmonary function studies, the operative findings, and biopsy of lung and heart. The results of the aminophyllin test were analyzed in the light of the postoperative re-evaluation, which included cardiac catheterization in several of these patients, performed at least six months after operation. We selected aminophylline because of its known effects on the circulation and respira-

From the Cardio Pulmonary Laboratory, Pathology Institute and the Thoracic Surgical Department of the Beilinson Hospital, Sick Fund of the General Federation of Labour, Israel.
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tion.[4-8] Other pharmacologic agents have been shown to produce changes on the pulmonary circulation.[2, 9, 10] Especially instructive is the study of the effects of aminophylline on the pulmonary circulation in patients with systemic hypertension.[8] Nevertheless, we thought that mitral stenosis, by narrowing the pulmonary circulation at its outlet, offers a unique opportunity to study the effects of aminophylline more specifically upon the smaller circuit. On the other hand, our cases allowed us to interpret in vivo these effects in the light of the biopsy findings obtained at operation. The operations were performed by Dr. L. Langer.

MATERIAL AND METHODS

The studies were performed on 10 patients with mitral valvular disease selected at random from the group being evaluated for mitral valvotomy. At the time of catheterization, no patient was in overt cardiac failure. Catheterization of the heart was carried out according to established principles.[11] In most of the cases, a double lumen catheter was used in order to obtain simultaneous pressures at two different points, mainly in the "pulmonary capillaries" and in the pulmonary artery, or in the pulmonary artery and in the right ventricle. Intra-arterial systemic pressure was always measured simultaneously with the pulmonary artery pressure. After control pressures and cardiac output were obtained, a single dose of aminophylline, 0.24 Gm., was given intravenously and all the measurements repeated at intervals of 15 minutes. At the moment of maximal drop of pulmonary artery pressure, after administration of aminophylline, values of cardiac output, pressures, and resistances were redetermined.

RESULTS

We studied the effects of aminophylline on various hemodynamic factors: systemic, pulmonary artery and "pulmonary capillary" pressures, systemic and pulmonary resistances, cardiac index, right ventricular work against pressure, right ventricular end diastolic pressure, and arterial oxygen saturation. The changes were of no statistical significance when each of these factors was considered separately for the entire group of patients. On the other hand, when the over-all effects of aminophylline were examined in each patient they gained meaning (Table IV).

Table I summarizes the most important clinical findings. The patients ranged in age between 21 and 41. The duration of the illness ranged from two years to ten years. Most of the patients were in Group II or III, according to the New York Heart Association Functional Classification. The ECG findings were typical of predominant or pure mitral stenosis. Roentgenologic findings were standardized for comparison, and all were compatible with mitral valvular disease. The preoperative clinical diagnosis was either predominant or pure mitral stenosis in all the patients sent to operation.

Table II shows the effects of aminophylline on the pressures and resistances of the pulmonary and systemic circulation. It can be seen they were not interdependent in direction or magnitude.

The pulmonary ventilatory function (Table III) before operation should be considered separately in every case. No consistent pattern was obtained, aside from the diminished breathing reserve at exercise which is practically a constant finding in mitral stenosis.[12] Individual cases, like No. 6, showed a

more definite and uniform pattern of pulmonary insufficiency because of added chronic pulmonary emphysema not related to the mitral disease. No attempt to evaluate the effects of aminophylline on pulmonary functions in these patients was done.

TABLE I. PREOPERATIVE CLINICAL STATUS

NO.	AGE	ILLNESS DURATION	DEGREE OF INCAPACITATION	ECG	ROENTGEN		CLINICAL DIAGNOSIS
					HEART	LUNG MARK.	
1	22	10 Y	III	R. A. D. R. V. H. + + Mitral P.	L. A. + + + R. V. + + L. V. + P. A. + +	+ + +/ + + + +	M. stenosis
2	21	8 Y	II-III	Mitral P.	L. A. + + P. A. + + R. V. +	+	M. S.
3	31	10 Y	III	R. A. D. A. F. R. V. H. + +	L. A. + + R. V. + + P. A. +	+ 	M. S.
4	22	3 Y	II	R. A. D. Mitral P.	L. A. + + + R. V. + + L. V. +	+ + +	M. S.
5	22	6 Y	II	Mitral P.	L. A. + + R. V. +	+	M. S.
6	33	10 Y	III	R. A. D. Mitral P. R. V. S. + +	L. A. + + R. V. + + L. V. + (?)	+ + +	M. S. Emphysema bronchiectasis
7	21	6 Y	II	R. A. D. R. V. H. + +	L. A. + R. V. +	+	M. S.
8	41	10 Y	IV	A. F. L. V. H. (?)	L. A. + + + R. V. + + + L. V. +	+ +	M. S. and ins.
9	34	4 Y	III	Mitral P.	L. A. + + R. V. + + L. V. + (?)	+ + +	M. S.
10	37	2 Y	II-III	L. A. D. L. V. H. (?)	L. A. + L. V. +	+	M. I. + Slight S.

The intensity of the lung markings and the degree of individual chamber enlargement are expressed from + to + + + +.

TABLE II. EFFECTS OF AMINOPHYLLINE ON PULMONARY AND SYSTEMIC PRESSURES AND RESISTANCES

CASE	MEAN PRESSURES (PER CENT OF CHANGE)		RESISTANCES (PER CENT OF CHANGE)		
	PULM. ART.	SYST. ART.	PULM. ARTERIOLAR	TOTAL PULM.	SYSTEMIC
1	+ 5	−20		−38	−53
2	−16	− 7	−50	+ 8	+19
3	+ 4	+25	−33	+13	+36
4	−10	−11	− 8	−20	−15
5	−33	No change	−60	− 4	No change
6	−31	No change	−60	−60	−41
7	−16	No change	−25	−44	−22
8	−16	No change	+ 7	+67	+43
9	−22	No change	−37	−35	−13
10	−31	−10	−46	−38	−23

In evaluating the lung and heart biopsies (Dr. N. Rosen), we have tried to assess separately the findings in the alveoli, the state of the bronchial

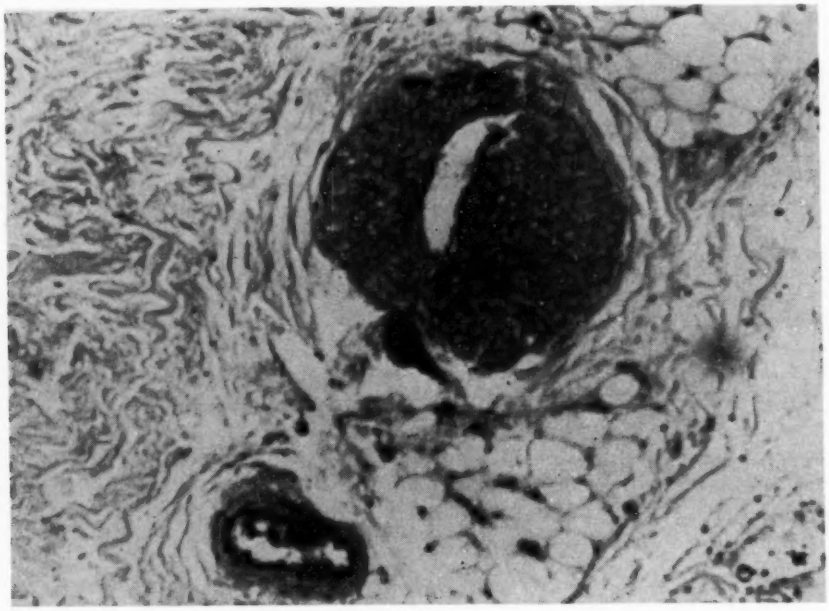

Fig. 1.—Elastic stain (magnification ×100). Blood vessels with hypertrophic musculature in pericardium.

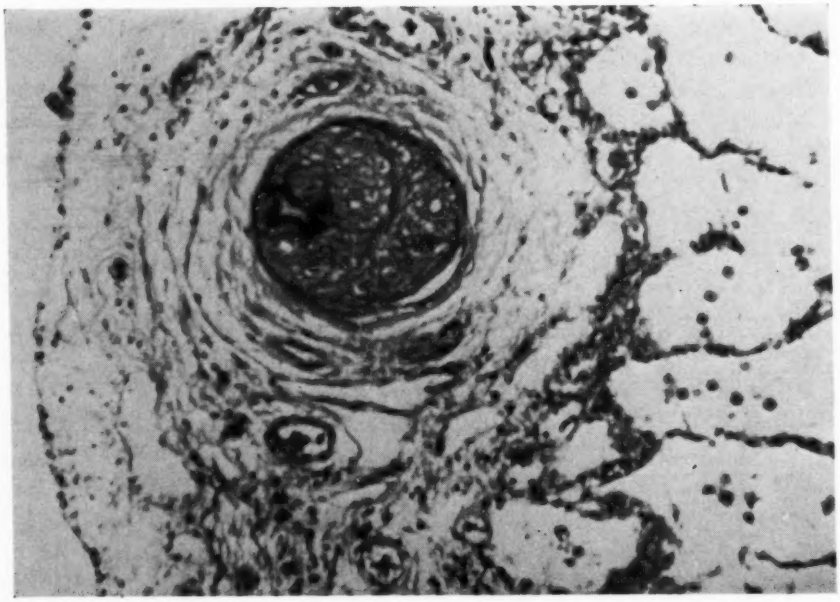

Fig. 2.—Elastic stain (magnification ×100). Blood vessel with hypertrophic musculature in pleura.

musculature and of the pulmonary arterioles and veins. Special interest was aroused by the presence of "abnormal blood vessels," regarding their morphol-

TABLE III. VENTILATORY FUNCTION

	M. B. C. (% OF NORM)	V. C. (% OF NORM)	V. C. T. (SECONDS)	BR. RES. (REST %)	BR. RES. (EX. %)	O_2 REMOVAL (ML./L.)
1	93	112	9	88	73	20
2						
3	61	69	9	89	72	45
4	93	55	6	89	75	26
5	119	127	13	95	92	55
6	47	68	7	79	51	30
7	122	64	4	87	76	35
8	89	100	6	90	79	24
9						
10	97	110	11	90	80	31

ogy and localization. These vessels, apparently small veins, were singularly located in the pleura and pericardium and were characterized by a remarkably increased musculature distributed in a nodular pattern along the length of the vessels and narrowing their lumen at the points of muscular hypertrophy (Figs. 1 and 2). They are absent in normal lungs or hearts. In almost every case where these blood vessels were found, the arteries and veins in the vicinity of the bronchi were found to be tortuous and thickened either by muscular hypertrophy or sclerosis. The presence of these blood vessels is not related quantitatively to the extent of lung fibrosis, and sometimes they were found in cases without lung fibrosis at all. The presence of these vessels in such a great number in the pleura, pericardium, and around the bronchi may represent the microscopic expression of an enlarged venous system. The macroscopic existence of a collateral bronchial venous system has been confirmed experimentally,[13] as well as in patients dying from mitral stenosis[14] and pulmonary emphysema.[15]

DISCUSSION

Only the cases with postoperative catheterization are included in the discussion and we intend to analyze the cases individually before arriving at conclusions (Table IV). Therefore, the cases were classified into three groups: (A) Cases where aminophylline preoperatively induced a beneficial hemodynamic response, similar to that obtained by commissurotomy as judged by the postoperative studies. (B) Cases where aminophylline preoperatively did not affect significantly the hemodynamic status and the patients also showed a lack of improvement after operation. (C) Cases where the preoperative effects of aminophylline did not parallel the postoperative results.

Group A.—Case 9: The patient was a 34-year-old woman classified in Group III, functional classification. Slight ECG and moderate x-ray changes were found. The preoperative aminophylline test showed a significant drop of pulmonary artery and "pulmonary capillary" pressures, an increase in cardiac output, and a drop in pulmonary resistances. At the same time, the right ventricular work and the end diastolic pressure were diminished. At operation, the mitral valve was very tight. The examination of biopsy material showed slight fibrosis, peribronchitis, increased bronchial musculature, muscular hypertrophy of arteries, and tortuous and thickened vessels in the pleura. Clinical

TABLE IV. COMPARATIVE DATA BEFORE AND AFTER OPERATION

CATHETERIZATION

NO.	PULMON. ART. MEAN PRESS. (MM. HG.)			PULM. CAPIL. MEAN PRESS. (MM. HG.)			RESISTANCES (DYN./SEC./CM.-5) PULM. ARTERIOL.			RESISTANCES (DYN./SEC./CM.-5) TOTAL PULMONARY			CARD. INDEX (L./MIN./M_2)		
	PRE	AM	POST	PRE	AM	POST	PRE	AM	POST	PRE	AM	POST	PRE	AM	POST
1	38	(40)	43			23			340	1050	(650)	730	1.75	(3.36)	2.76
2	48	(40)		26	(32)		360	(180)		830	(900)		3.40	(2.70)	
3	50	(52)		21	(30)		750	(505)		1220	(1375)		2.35	(1.96)	
4	50	(45)		26	(21)		500	(460)		1000	(800)		2.80	(3.00)	
5	30	(20)	14	23	(18)	8	88	(36)	102	376	(360)	235	3.70	(3.00)	2.60
6	65	(45)	60	27	(18)	30	675	(280)	430	1150	(470)	860	2.62	(4.50)	3.30
7	38	(32)	16	27	(20)	10	220	(164)	125	770	(430)	330	2.60	(3.40)	2.45
8	43	(36)	42	32	(28)	32	240	(257)	240	900	(500)	1000	2.61	(1.72)	2.11
9	55	(43)	20	30	(23)	18	510	(320)	385	1113	(765)	480	2.65	(3.00)	2.74
10	19	(13)		9	(7)		185	(100)		350	(216)		2.80	(3.10)	

Pre = Preoperative catheterization.
Am = Aminophylline effects at preoperative catheterization.
Post = Postoperative catheterization.

follow-up and postoperative catheterization showed improvement which paralleled the preoperative aminophylline response even to a greater degree. In this case, the improvement of the hemodynamic status of the pulmonary circulation is evident.

Case 5: The patient was a 22-year-old man, classified in Group II, mild ECG and x-ray findings. On preoperative catheterization, aminophylline effected a drop of pulmonary artery and "pulmonary capillary" pressures and resistances. The most significant change was the drop of the arteriolar resistance. A marked diminution of right ventricular work, along with an increase in oxygen saturation, was also noted. At operation, a tight mitral stenosis was found. The examination of biopsy material showed: slight muscular hypertrophy of arteries and slight venous congestion. The postoperative catheterization showed a drop in pressures and resistances more marked than those achieved by aminophylline preoperatively. This is compatible with the slight changes found at the biopsy.

Case 7: The patient was a 21-year-old woman, classified in Group III. Slight ECG and moderate x-ray changes were present. Preoperative catheterization showed that aminophylline induced a significant drop of pulmonary resistances and a less marked decrease of pulmonary artery and "pulmonary capillary" pressures, in the presence of a significant increase of cardiac index and arterial oxygen saturation. At the same time a very significant drop of right ventricular work took place. At the operation, the mitral valve was very tight. The biopsy examination showed moderate fibrosis. Postoperative catheterization demonstrated a significant hemodynamic improvement, much more pronounced than that obtained by aminophylline, and the clinical status was very much improved. In this case, we assumed the presence of a pure arteriolar constriction which was ameliorated by aminophylline and completely relieved by the satisfactory opening of the mitral valve.

Group B.—Case 8: The patient was a 41-year-old woman, classified in Group IV. ECG signs pointed to left ventricular predominance, and x-ray

INCLUDING RESULTS OF PREOPERATIVE AMINOPHYLLINE TEST

DATA										FOLLOW UP
ART. O_2 SATURATION			RT. VENTR. WORK (KG./M./MIN./M_2)			END DIAST. PRESSURE (MM. HG)			OPERAT.	DEGREE OF CLINICAL IMPROV.
PRE	AM	POST	PRE	AM	POST	PRE	AM	POST	MITRAL VALVE	
93	(95)	95	0.90	(1.77)	1.55			3	V. T.	+
90	(93)		1.97	(1.31)		5	(5)		Not operated yet	
95	(98)		1.61	(1.40)		4	(4)		V. T.	+(?)
96	(98)		1.90	(1.84)		1	(1)		V. T.	++
96	(98)	98	1.54	(0.81)	0.95	2	(2)	4	T.	+++
88	(91)	94	2.40	(2.80)	2.46	10	(5)	10	V. T.	+
94	(96)	98	1.30	(0.39)	0.38	4	(2)	0	V. T.	+++
91	(92)	94	1.34	(0.71)	0.62	6	(6)	10	Sten. + insuff.	=
94	(88)?	99	1.80	(1.50)	0.59	8	(7)	6	V. T.	+++
96	(96)		0.65	(0.53)		2	(3)		Not operated	

T. = Tight.
V.T. = Very tight.

examination showed moderate changes in the pulmonary markings and right ventricular enlargement. On catheterization, the elevated pressure in the pulmonary artery and "pulmonary capillaries" were slightly lowered after aminophylline while the arteriolar resistance increased. Cardiac index dropped even further. At operation, slight mitral stenosis and insufficiency was found. The biopsy examination showed a marked sclerosis of most of the bigger pulmonary vessels, moderate fibrosis and emphysema, and slight sclerosis of most of the small vessels. In the pleura, blood vessels with hypertrophied musculature were found. After operation, the patient's status did not change. Postoperative catheterization: the findings were practically the same. Clinically, no improvement was noted.

At this stage, the increased pulmonary resistance is no longer a result of the mitral disease, but of the anatomically fixed changes in the pulmonary circulation which neither aminophylline nor the valvotomy can change.

Group C.—Case 1: The patient was a 22-year-old man, classified in Group III. Moderate ECG and marked x-ray changes were found pointing to pulmonary engorgement. On preoperative catheterization, aminophylline produced a diminution of the total pulmonary resistance, and the cardiac output increased. However, it is to be noted that the pulmonary artery pressure was not changed, while the right ventricular work increased markedly. In this case, the diminished pulmonary resistance may be partially the results of the simultaneous marked drop in the systemic resistance (–53 per cent, see Table II). At operation, the mitral valve was very tight. The biopsy examination showed slight emphysema, marked fibrosis, thickening of the intima of the arterioles, enlarged veins, and increased number of blood vessels in the pleura and pericardium. Clinically, there was slight improvement after operation, but the patient suffered from recurrent episodes of right heart failure. Postoperative catheterization: the results paralleled the aminophylline response preoperatively, showing no drop whatsoever in the pulmonary artery pressure. It should be emphasized that the patient had marked pathologic changes in the lungs leading to cor

pulmonale. The fact that the pulmonary artery pressure did not drop after aminophylline may serve as a prognostic guide in the poor response of this type of case.

Case 5: The patient was a 33-year-old woman, classified in Group III. Moderate ECG and x-ray changes were found. At catheterization, aminophylline induced a marked drop of pulmonary resistances, pulmonary artery and "pulmonary capillary" pressures, and a marked increase in cardiac output and significant drop of the end diastolic pressure of the right ventricle. At operation, a very tight mitral stenosis was found. The biopsy examination revealed slight fibrosis, marked emphysema, marked sclerosis of arterioles and veins, "abnormal blood vessels" in the pericardium, and increased bronchial musculature and bronchitis. Clinically, there was slight improvement. Postoperative catheterization showed a drop of pulmonary resistances similar to that effected by aminophylline preoperatively but to a much lesser degree, while the pulmonary artery and "pulmonary capillary" pressures were essentially unchanged. Oxygen saturation remained improved. The increase in cardiac output following commissurotomy accounted for the clinical improvement, which remained incomplete because of the presence of vascular changes on the basis of long-standing bronchial disease. The patient's pulmonary function studies showed definite signs of ventilatory insufficiency; this feature helps to differentiate mitral stenosis with true emphysema from the occasional patient with mitral stenosis suffering from asthmalike attacks and who following successful commissurotomy is completely relieved from "asthma."[12]

Cases 2, 3, 4, and 10 are not presented because no second catheterization has been carried out as yet.

COMMENTS

We expected that aminophylline would serve as a guide to classify preponderant reversible hemodynamic changes versus irreversible changes in the pulmonary circulation. In that sense, a significant beneficial response to aminophylline preoperatively, which was confirmed in the postoperative studies by a parallel improvement, is as reliable a result as that in which aminophylline did not elicit a favorable response and follow-up studies did not show appreciable improvement.

1. The cases in which the lung biopsies did not show extensive pulmonary damage consistently responded to aminophylline trial with a decrease in pulmonary resistances, more marked in the arteriolar than in the total resistance. The operation, as judged by the control catheterization, achieved the same or even better effects.

2. The best correlation was obtained when aminophylline equally diminished both pulmonary resistances and pressures. Whenever only the resistances dropped, the postoperative results were not as good and meant the presence of a significant amount of anatomical damage (Case 1).

3. It is apparent in the majority of cases that the decrease of pulmonary pressures and resistances, in the presence of mitral stenosis, are independent of similar simultaneous changes in the systemic circulation.

4. The effect of aminophylline is characterized by a decrease of the pulmonary arteriolar resistance which should be accompanied by an increase in cardiac output. It is worth-while to determine whether this is accomplished by either: (1) dilatation of the small arterioles, or (2) by a decrease of the "bronchomotor tone."[16] Simultaneous measurement of the effect of aminophylline upon the pulmonary artery and bronchial pressures would help to clarify this. A change in the tonus of pulmonary veins should also be considered.

5. The hypothesis brought forward is that the special blood vessels described in the biopsy findings could represent the histologic picture of collateral bronchial venous circulation. Macroscopically, this has been proved repeatedly in different disease states[15, 17, 18] and also experimentally.[13] We agree with Ferguson[14] that in advanced cases of mitral stenosis, the patients may develop a reversed flow shunt, via the left auricle, pulmonary vein, bronchial vein, azygos, and back to the right auricle. We think that this collateral "third circulation" may play an important role in mitral stenosis as a safety valve reservoir, by diverting blood from the already engorged pulmonary venous system and serving to protect the pulmonary capillaries from being overflooded. It remains to be explained which patients and under which particular circumstances are prone to develop collaterals. This may be particularly important in the hemodynamic adjustment of the patient with mitral stenosis and we plan to study it further with the use of radioactive material injected in the pulmonary circulation.

As yet, the mechanism by which aminophylline induced a decrease of "pulmonary capillary" pressure is not readily apparent. As the size of the mitral valve is fixed, two possibilities can be entertained: (1) Aminophylline dilated the hypothesized collateral circulation. (2) A decrease of the venomotor tone. In any case, the net result should be that the inflow to the left auricle is lessened or directed under lower head pressure.

The aminophylline trial should be reserved for the preoperative evaluation of long-standing mitral stenosis or when there are signs of cor pulmonale. These are the cases where a preoperative evaluation is more difficult.

SUMMARY

A single dose of intravenous aminophylline was administered at the time of preoperative cardiac catheterization to patients being evaluated for mitral commissurotomy. Correlation of its hemodynamic effects with the results of operation permitted us to draw certain conclusions as to its reliability as a prognostic guide in the selection of patients for valvotomy. The results of the aminophylline test were also correlated with the lung biopsy findings.

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SERUM CONCENTRATIONS OF VITAMIN B_{12} IN ACUTE AND CHRONIC LIVER DISEASE

M. RACHMILEWITZ, M.D., J. ARONOVITCH, M.Sc., AND N. GROSSOWICZ, PH.D.
JERUSALEM, ISRAEL

IN STUDIES on the determination of serum levels of vitamin B_{12} and its application to clinical problems,[1, 2] we noted a definite rise in serum B_{12} in cases of cirrhosis of the liver. Further investigation showed this rise of serum B_{12} to occur in other types of liver disease as well, and a preliminary report has been published.[3] In the following communication this study has been extended to include serial examinations of serum B_{12} in cases of acute and chronic liver disease.

EXPERIMENTAL PROCEDURE

Microbiologic Assays.—The vitamin B_{12} determinations were carried out by the modified *Escherichia coli* assay.[1, 2] Serum values in 25 healthy individuals ranged from 200 to 500 $\mu\mu$g per milliliter with a mean of 340 $\mu\mu$g per milliliter. In most cases repeated B_{12} assays were carried out during the course of the disease. The results were correlated with the usual liver function tests and electrophoretic pattern of the serum proteins.

The total serum B_{12} was determined after the serum diluted 1:8 with distilled water was heated for 30 minutes at 100° C. and added aseptically to the medium. When the B_{12} was liberated from the serum proteins by acid precipitation (pH 5.5) according to the method of Spray,[4] nearly the same results were obtained as without precipitation. Free serum B_{12} was estimated in the unheated sample.

In the determining of the maximum binding capacity (MBC) of serum for vitamin B_{12}, 1 ml. of serum was mixed with 0.5 ml. of distilled water containing known amounts of crystalline B_{12} and incubated at 4° C. for 24 hours. The serum was then reassayed for bound and free B_{12}. The MBC was determined by using at least 2 different concentrations of B_{12}, usually 1,500 and 3,000 $\mu\mu$g. In 10 normal individuals, in whom the total B_{12} ranged from 200 to 500 $\mu\mu$g per milliliter, the MBC was in the range of 1,200 to 2,000 $\mu\mu$g per milliliter. The recovery was 80 to 110 per cent of the added vitamin. When the serum with added B_{12} was incubated for 1 hour instead of 24 hours, the MBC was found to be lower, approximately 1,000 $\mu\mu$g per milliliter.

Since *E. coli* responds to methionine as well as to vitamin B_{12}, control assays were done on sera treated with sodium hydroxide (final concentration 0.4 N) to destroy the B_{12}. The samples were steamed for one hour, neutralized, and reassayed. Practically no growth-promoting activity was found in the sera after this treatment.

RESULTS

Acute Hepatitis.—In 13 out of the 14 patients with acute viral hepatitis the initial B_{12} of the serum was high, ranging from 800 to 6,000 $\mu\mu$g per milliliter, far above the normal range (Table I). These patients showed various degrees

From the Departments of Internal Medicine "B" and Bacteriology, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

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TABLE I. SERUM B_{12} CONCENTRATIONS, CAPACITY TO BIND B_{12}; LIVER FUNCTION TESTS AND ELECTROPHORETIC PATTERN IN ACUTE HEPATITIS

PATIENT	DATE	TOTAL B_{12} ($\mu\mu$g/ML.)	FREE B_{12} ($\mu\mu$g/ML.)	MAXIMAL BINDING CAPACITY FOR B_{12} ($\mu\mu$g/ML. SERUM)	BILIRUBIN (MG. %)	LIVER FUNCTION TESTS			TOTAL PROTEIN (GM. %)	ELECTROPHORESIS				
						CEPHALIN FLOCCULATION	THYMOL FLOCCULATION	THYMOL TURBIDITY (UNITS)		ALBUMIN (%)	GLOBULIN			
											$ALPHA_1$ (%)	$ALPHA_2$ (%)	BETA 1 + 2 (%)	GAMMA (%)
1. M. K.	3/21/55	1,400	385	2,250	17.9	+	+	7.3	6.2					
M. K.	3/28/55	850	135	1,900	9.8	−		3.7	5.7	29.1	8.2	17.3	21.5	23.5
M. K.	4/18/55	750			2.1					29.2	12.2	12.3	19.4	21.9
M. K.	5/18/55	600			0.9	−		2.4		52.0	2.0	8.0	18.0	20.0
M. K.	6/28/55	290	<50	1,500										
2. B. Sh.	5/23/55	800	0	1,300	7.2	+	+	7.2	6.3	51.0	4.0	5.0	18.0	22.0
B. Sh.	5/ 6/55	830	100	1,000	3.2	+	+	4.7						
3. M. G.	8/26/55	840	0	2,000	8.5	++		4.3	5.3	33.3	11.1	8.8	15.1	30.8
4. J. B.	8/14/55	940		1,650	12.9	+++	+++	13.3		27.7	8.4	17.4	18.8	29.0
J. B.	11/ 1/55	720	0							39.4	6.8	12.6	13.3	28.7
5. H. Sh.	9/23/55	3,350	150	3,800	7.5	++	++	15.1	6.3	43.4	4.7	10.0	17.7	32.2
H. Sh.	10/ 2/55	1,780	175	2,050	8.5									
H. Sh.	10/ 6/55	1,680	0	2,250	6.6	+++		11.7						
H. Sh.	10/12/55				3.8	+++	++	18.0						
H. Sh.	10/17/55	700		1,700						31.2	5.6	12.1	17.1	36.4
6. C. A.	10/12/55	2,500	100	2,660	13.5	+		10.0	7.3	41.8	6.2	7.1	14.8	30.0
C. A.	10/17/55	2,550	125	2,750										
C. A.	10/22/55				27.4	+++		19.6						
C. A.	11/ 2/55	860			8.3					31.0	8.2	13.5	13.5	34.7
C. A.	11/ 7/55	735	0	1,750	7.5	++		16.3		33.6	7.3	11.8	14.4	32.7
7. D. G.	10/18/55	2,350	100	3,300	6.6	++	++	14.3		54.6	2.2	5.0	9.8	29.8
D. G.	10/20/55				11.4	++	++	19.7						
D. G.	10/26/55	875	0	2,300										
D. G.	10/28/55				4.1	+	++	20.0	8.1	40.6	3.6	11.7	15.3	28.8
D. G.	10/31/55				3.25	+++	+++	18.8	8.7					
D. G.	11/20/55	425		2,100	0.9	+++	+++	14.1	7.9					
8. A. Kh.	10/24/55	1,425	250	2,750		+		5.7	6.3	30.4	9.4	12.9	16.1	32.8
A. Kh.	10/27/55				15.5	−		4.2	5.7					
9. O. S.	11/ 6/55	1,650			12.6		+++		7.2	34.2	6.4	13.9	20.3	25.2
O. S.	11/20/55	760			7.9	+++	+++	14.2		28.2	7.9	10.0	15.1	38.8
10. G. R.	12/ 8/55	2,150			13.9	++++	++++	22.9	6.4	27.9	4.9	3.5	5.6	57.7
G. R.	12/18/55	620			8.2	+++	+++	20.4		33.6	4.0	16.4	14.1	31.9
G. R.	12/25/55	470			3.5	+++	+++	17.8						
11. B. S.	12/13/55	1,150			12.8	+++	+	6.7	7.0	35.3	4.9	7.3	19.1	33.1
B. S.	12/19/55	640			16.2	+++	++	13.0	7.4	34.1	6.2	15.5	16.8	27.4
B. S.	1/21/56	300			2.4			6.3	6.8	43.7	4.5	11.6	16.3	24.6
12. T. G.	12/15/55	400			19.1	++		5.0	5.4	30.3	6.0	10.0	11.9	41.8
T. G.	12/23/55	200			6.7	+								
13. M. Wa.	12/27/55	1,565			17.5		+	9.0	6.8	32.7	6.6	10.9	19.0	31.7
M. Wa.	1/ 9/56	1,000			21.2					39.7	7.5	10.9	22.2	18.9
14. L. W.	1/13/56	6,000	150		9.9	+++	+++	21.0		30.0	8.2	9.0	12.3	39.3
L. W.	1/21/56	2,100	100		7.8			20.2		33.9	6.9	10.0	11.3	37.8

TABLE II. SERUM B_{12} CONCENTRATION, CAPACITY TO BIND B_{12}; LIVER FUNCTION TESTS AND ELECTROPHORETIC PATTERN IN CIRRHOSIS OF THE

TABLE II. SERUM B_{12} CONCENTRATION, CAPACITY TO BIND B_{12}; LIVER FUNCTION TESTS AND ELECTROPHORETIC PATTERN IN CIRRHOSIS OF THE LIVER

PATIENT	DATE	TOTAL B_{12} ($\mu\mu$g/ML.)	FREE B_{12} ($\mu\mu$g/ML.)	MAXIMUM BINDING CAPACITY MBC ($\mu\mu$g/ML. SERUM)	BILIRUBIN (MG. %)	LIVER FUNCTION TESTS: CEPHALIN FLOCCULATION	LIVER FUNCTION TESTS: THYMOL FLOCCULATION	LIVER FUNCTION TESTS: THYMOL TURBIDITY (UNITS)	TOTAL PROTEINS (GM. %)	ELECTROPHORESIS: ALBUMIN (%)	ELECTROPHORESIS: GLOBULIN: ALPHA$_1$ (%)	ELECTROPHORESIS: GLOBULIN: ALPHA$_2$ (%)	ELECTROPHORESIS: GLOBULIN: BETA 1 + 2 (%)	ELECTROPHORESIS: GLOBULIN: GAMMA (%)	REMARKS
1. H. G.	4/30/54	1,175			2.8	+	+	7.6	5.3						Portal cirrhosis in decompensation
2. F. D.	12/23/54	620			1.7	++	++	19.0	5.5						Portal cirrhosis and nephrosis
3. J. Sh.	5/ 3/55	830			4.3	+	+	8.0	6.4	26.9	9.0	16.6	9.3	37.9	Biliary cirrhosis
4. T. M.	1/ 2/55	1,350	250	2,100		+	+	14.6	6.5	30.0	7.3	7.0	15.5	39.7	Portal cirrhosis in decompensation
T. M.	3/10/55				3.4	+	+	7.1	6.7						
T. M.	4/18/55	1,250	300	2,500						20.5	6.5	7.0	14.5	50.3	
5. A. Z.	7/ 4/55	3,600	1,500	2,100	16.0	+	+	7.8	6.9	54.5	3.7	10.4	6.9	24.5	Biliary cirrhosis
6. J. A.	10/ 6/55	1,600	50	2,000	6.9	+++	+	10.0		34.0	3.9	10.5	14.7	23.5	Biliary cirrhosis + hepatoma
J. A.	10/12/55					+++	++	15.2							
J. A.	10/17/55	1,750			11.0			14.3		36.2	6.7	11.9	14.8	30.0	
J. A.	10/27/55				15.8	++	+	8.7		25.2	8.0	15.7	20.5	30.2	
J. A.	10/30/55	1,200	100	2,500											
7. J. Am.	10/16/55	1,250		2,000	0.8	+++	+++	17.0	7.8	36.3	8.9	13.1	12.2	30.9	Portal cirrhosis in decompensation
J. Am.	10/19/55					+	++	11.7							
J. Am.	10/23/55	1,000				−	++	14.1							
J. Am.	10/27/55	1,050													
J. Am.	10/31/55					+	+	7.5	7.1	39.0	3.3	8.9	12.2	36.6	
J. Am.	11/ 3/55	800	75	1,850						25.9	3.5	11.3	16.4	41.6	
8. E. I.	11/ 5/55	250			2.1	+	+	6.0	7.0	29.6	9.8	12.6	19.5	29.6	Portal cirrhosis in compensation
9. M. B.	12/12/55	1,800			8.2	−		2.4	7.2	30.0	3.2	11.5	23.5	32.0	Biliary cirrhosis
10. H. W.	12/16/55	625			3.2	+		4.8	6.2	29.0	2.5	1.7	66.6		Portal cirrhosis in decompensation
11. S. H.	1/ 9/56	1,900			11.8	−		3.8	7.1						Biliary cirrhosis

of hepatocellular damage as manifested by liver function tests. During the course of the disease there was a gradual decrease in the B_{12} level with the improvement in the clinical condition and subsidence of jaundice. No correlation was found between the intensity of the jaundice and the level of B_{12} in the serum. The highest values of B_{12} were found in the serum of 5 patients (Cases 5, 6, 7, 10, and 14) with viral hepatitis in the early phase of the disease. The lack of correlation between the intensity of jaundice and the B_{12} level is particularly striking in this phase. Liver function tests were still abnormal when B_{12} concentrations were approaching normal levels.

In 8 patients (Cases 1 to 8) free and bound B_{12} of the serum were examined. It was found that practically all of the vitamin was in the bound form. The maximal binding capacity (MBC) of the serum in these cases ranged from 1,000 to 3,800 $\mu\mu$g per milliliter. The highest values were obtained in cases which were examined early in the disease at the time when the total B_{12} was very high. The decrease in B_{12} levels in the course of the disease was associated with a decrease of the MBC.

Cirrhosis of the Liver.—Eleven patients were examined, 5 of whom had biliary cirrhosis and 6 had portal cirrhosis. In the 5 patients with biliary cirrhosis the serum B_{12} concentrations were high (830, 1,600, 1,800, 1,900, and 3,600 $\mu\mu$g per milliliter) (Table II). These patients were jaundiced and with laboratory examinations indicative of liver damage. In 3 of 6 patients with portal cirrhosis serum B_{12} was high (1,175, 1,250, and 1,350 $\mu\mu$g per milliliter); in the remaining 3 it was normal. In 2 of the latter, liver function was normal (Cases 8 and 10); the third (Case 2), with positive liver function tests, had nephrosis in addition to portal cirrhosis, verified at autopsy. The MBC of the serum of the patients with cirrhosis ranged from 1,850 to 2,500 $\mu\mu$g per milliliter.

Extrahepatic Obstructive Jaundice.—In 6 patients with jaundice due to obstruction of extrahepatic bile ducts by stones (3 cases) or by carcinoma of the head of the pancreas (3 cases), the vitamin B_{12} levels were within the normal range (300, 360, 380, 480, 510, and 660 $\mu\mu$g per milliliter).

Liver function tests (cephalin flocculation, thymol flocculation, and thymol turbidity) performed simultaneously with the B_{12} determinations were normal; however, alkaline phosphatase and cholesterol values were increased.

Electrophoresis.—The electrophoretic patterns of the sera of most cases with liver damage were characterized by decrease of the albumin fraction and increase of globulin, particularly of gamma globulin. This was true in the acute as well as in the chronic cases. The alpha fractions were elevated in some and normal in others. No correlation was found between the level of alpha globulins and B_{12} concentrations.

DISCUSSION

The present study shows that the serum vitamin B_{12} concentration is often markedly increased in liver disease and may be as high as 20 times the normal. This is particularly striking in acute viral hepatitis with severe liver damage. In 3 patients with acute hepatitis (Cases 2, 3, and 4), where the elevation was

not as marked, the B_{12} determinations were performed in the recovery stage of the disease. The only exception in this group was a patient (Case 12) with a clinical picture of viral hepatitis with high but transient bilirubinemia and with only slight abnormalities in other liver function tests.

In cirrhosis of the liver, the B_{12} findings were less uniform than in acute hepatitis, although the highest values were found in patients with severe liver damage. However, due to the limited number of observations it was not possible to seek correlation between the level of B_{12} in the blood, the type of cirrhosis, and the particular stage of the disease. Other investigators[5, 6] have mentioned finding elevated B_{12} levels in some cases of cirrhosis of the liver.

The presence and intensity of jaundice were not correlated with the level of B_{12} in the blood. Thus, in some patients with cirrhosis of the liver high B_{12} values were obtained in the absence of jaundice. In acute hepatitis the highest B_{12} concentrations were found in the early phase of the disease before blood bilirubin reached the highest level.

The common denominator of the high B_{12} serum level in acute as well as in chronic liver disease is the presence of hepatocellular damage. Of particular significance are the findings of normal B_{12} values in 6 patients with jaundice due to obstruction of the extrahepatic bile ducts without laboratory evidence of hepatocellular damage. Our preliminary observations suggest that the determination of B_{12} in the blood might be helpful in differentiating hepatocellular from extrahepatic obstructive jaundice.

In myeloid leukemia the B_{12} values[6-9] are even higher than those reported here for liver disease. In liver disease, as in myeloid leukemia, most of the vitamin was in the bound form.[7-10] In a single case of biliary cirrhosis with very high serum B_{12} level, almost half of the vitamin was in the free form (Case 5, Table II).

In chronic myeloid leukemia the B_{12} is bound chiefly to the alpha globulin fraction.[7, 8, 10] Electrophoretic studies of the sera of our patients with liver disease showed the usual decrease in albumin and rise in globulin, particularly gamma globulin. Some sera showed increased alpha globulins, but there was no correlation between this rise of alpha globulin and the elevation of B_{12}.

The mechanism responsible for the elevation of serum B_{12} in liver disease with hepatocellular damage remains to be elucidated. It is possible that this increase may be due to changes of serum proteins resulting in increased capacity to bind B_{12}. Our determinations of the MBC are inconclusive in this respect. Another possible cause for the rise of the serum B_{12} may be reduced excretion of B_{12} by the kidney, which may lead to increased free B_{12} in the serum. Our findings do not support such an assumption, since most of the vitamin in the serum was bound. Last, a plausible cause of increased serum B_{12} in liver injury may be the release from damaged liver cells of stored B_{12}. The presence of liver damage in all cases with elevated B_{12} and the normal B_{12} values in obstructive extrahepatic jaundice are in favor of this assumption. In this case, the mechanism of the increase of B_{12} in the blood may be similar to that of the rise of serum iron observed in parenchymatous liver disease.[11]

SUMMARY

1. High vitamin B_{12} values were found in the sera of 13 out of 14 patients suffering from acute viral hepatitis. The concentrations ranged from 800 to 6,000 $\mu\mu$g per milliliter and decreased gradually with subsidence of jaundice.

2. In 5 cases of biliary cirrhosis and in 3 out of 6 cases of portal cirrhosis the B_{12} concentration was high and ranged from 830 to 3,600 $\mu\mu$g per milliliter.

3. In 6 cases with extrahepatic biliary obstruction and jaundice the serum B_{12} was within normal range.

4. The maximal binding capacity (MBC) of the serum of normal individuals ranged from 1,200 to 2,000 $\mu\mu$g per milliliter. In patients with liver disease and high vitamin B_{12} in the serum, the MBC was increased.

5. The increase of serum vitamin B_{12} in liver disease is considered to be due to the release of the stored vitamin from damaged liver cells.

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THE MACROCYTOSIS OF LIVER DISEASE

CHARLES A. HALL, M.D.
ALBANY, N. Y.

INTRODUCTION

ABNORMALITIES of the size or shape of erythrocytes may reflect abnormalities of cell maturation as in pernicious anemia, abnormalities of hemoglobin as in sickle-cell anemia, and, as suggested by recent studies of hereditary spherocytes,[1] abnormalities of cell metabolism. Possibly the macrocytosis often associated with liver disease also reflects a basic abnormality of the cell. Many investigators have described macrocytosis in patients with liver disease,[2-15] but the reported incidence has varied widely. Macrocytosis has been measured by determination of mean cell volume, by the diffraction technique of determining mean cell diameter, or by measurement of individual cells by one of the many, and sometimes inaccurate, techniques of direct cell measurement. Part of the variation in the reported incidence of macrocytosis resulted from differences in technique of measurement, and part was due to differences among the group of patients studied.

Larsen[8] reported a high incidence of macrocytosis in patients with hepatitis which he attributed to some defect of hematopoiesis. The results of his cell measurements could best be explained by assuming the production of two red cell populations, one distributed about a normal mean and the other distributed about a mean 0.75 μ to 1.0 μ greater than normal. If this distribution of red cells was due to the hepatic damage and was not merely the consequence of the particular type of liver disease studied, similar findings would be expected in patients with other types of liver damage. If the changes were fundamental abnormalities due to liver damage, the degree of abnormality of the erythrocytes would probably be related to the degree of abnormality of liver function regardless of its etiology.

The purpose of the present study was to determine if the type of macrocytosis described by Larsen was characteristic of other types of diffuse hepatocellular damage, and whether the macrocytosis was related to the liver damage or to some factor associated with liver disease such as the inadequate diet of patients with cirrhosis. Since the study was concerned with the relationship between liver function and both the morphologic and numerical changes in erythrocytes, nonanemic patients were not excluded. Patients whose anemia was due to hemorrhage or to some disease unrelated to the liver disease were excluded. Hypersplenism was a definite factor contributing to the red cell changes in some cases reported in the literature.[16, 17] Possible hypersplenism

From the Department of Medicine, Veterans Administration Hospital, Albany, N. Y.
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complicates any attempt to determine the effect of hepatic damage on red cell production or destruction since the spleen is frequently enlarged in patients with liver disease. Although splenic effects must be considered in the cases in the present study, none of the patients had obvious hypersplenism.

MATERIALS AND METHODS

A total of 35 patients was studied. Twenty-four patients had definite Laennec's cirrhosis. All cirrhotics who were admitted during a 10-month period were included, except those with a history of hematemesis and those with occult blood in the stool. Two patients with carcinoma of the lung, and severe renal disease, respectively, were also excluded. All were chronic alcoholics. Eighteen of this group were classified as having clinically significant liver disease, and 6 who had definite liver disease but of no clinical significance were classified as having mild cirrhosis. Five patients were severe, chronic alcoholics but had no clinical or laboratory evidence of hepatic disease. Three patients had either severe acute viral hepatitis or chronic recurrent hepatitis. None of the patients with hepatitis were alcoholics, and all had been receiving adequate diets. Two patients had huge livers filled with metastatic carcinoma but were otherwise in relatively good condition. One patient had a hepatoma without cirrhosis. The diagnosis in 10 patients of the entire group was confirmed by liver biopsy, and the diagnosis in 6 others was proved at autopsy.

Initial hematological studies consisted of red blood count, hemoglobin, hematocrit, reticulocyte count, while blood count, differential, platelet count, and prothrombin time—all performed in duplicate—and bone marrow examination. The red blood count, hemoglobin, hematocrit, and reticulocyte count were repeated weekly or biweekly, depending on the clinical status of the patient. The mean diameter (MD) was determined on admission and at appropriate intervals thereafter for a total of 1 to 4 determinations for each patient. There was a total of 59 MD determinations on the 35 patients. Hemoglobin was measured by a photoelectric colorimeter by an alkaline hematin method. A vital staining technique was used for the reticulocyte count. Red cell diameters were determined by the direct photographic method of Larsen.[8] The prothrombin time was performed by both the method of Quick[18] and of Owren.[19]

Total and partitioned serum bilirubin, 45-minute bromsulfalein, thymol turbidity, cephalin flocculation, and alkaline phosphatase were determined on admission, and weekly or biweekly thereafter. Serum albumin, serum globulin, total cholesterol, cholesterol esters, and alkaline phosphatase were determined on admission and then at monthly intervals. Only 1 or 2 sets of hematological and liver function studies were possible in the patients who had little disease and did not remain in the hospital. Patients with severe or prolonged illness were studied over periods of 1 to 6 months.

RESULTS

Normal Subjects.—The average mean diameter of the erythrocytes of the 16 normal subjects and other hematological data for 11 of these are given in Table I and Fig. 1. The frequency distribution curves of the 200 cells measured in each case were of normal configuration.

Significant Cirrhosis.—The 18 patients with clinically significant cirrhosis are considered separately. The average MD was within the upper range of normal in 4 cases. As illustrated in Fig. 2, the lower range of individual cell size was about the same as for the normal group, but cells larger than normal were present. Almost all of the patients had cells more than 9.25 μ in diameter. In one case, 73 per cent of the cells were larger than 9.25 μ, and cells larger than 11.00 μ were present. While the average red blood count of the group was below normal, 2 patients had counts of over 4.5 million, and 5 between 4.0 M and

4.5 M. All of these patients had macrocytosis. The average reticulocyte count was above normal, the highest count being 12.2 per cent. It was 2 per cent or below in only 3 instances, and one of these 3 cases had the greatest MD, 9.73 μ.

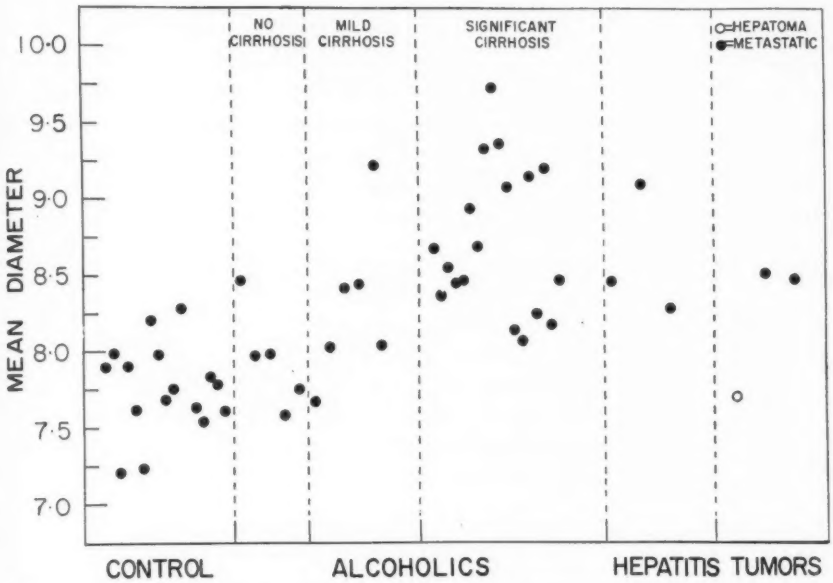

Fig. 1.—Mean diameters of normal subjects and the initial mean diameter of each patient.

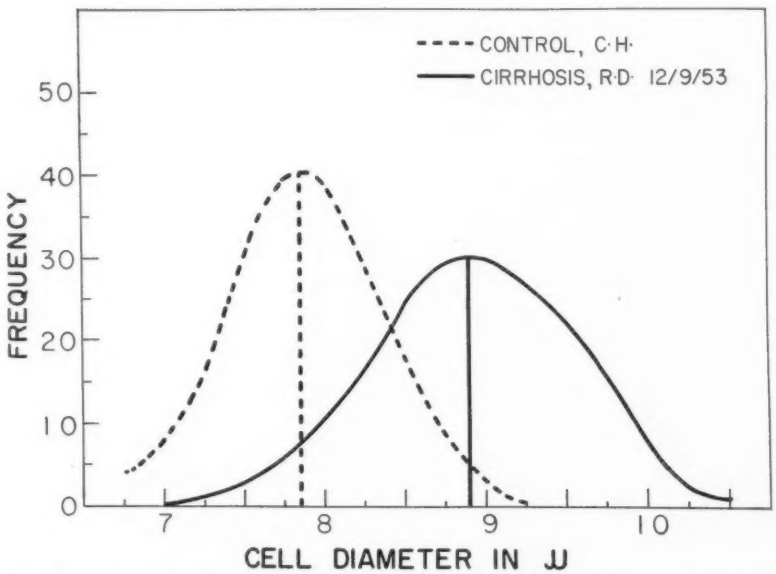

Fig. 2.—Frequency distribution curves of a normal subject and a patient with cirrhosis. Vertical lines indicate the mean diameter of the 200 cells.

The frequency distribution curves were distinctly abnormal in all cases. Sixteen of the initial curves were skewed in varied manners and degrees as

illustrated by the curve of Patient R. D. in Fig. 2. Two of the curves were of normal configuration, but the entire curve was shifted to the right. The analysis of the curves will be discussed below.

The average mean corpuscular volume of the group was above normal, 105 cu. μ, but the change in cell volume was not as pronounced as the change in cell diameter.

Mild Cirrhosis.—The erythrocyte changes of the 6 patients with mild and clinically insignificant cirrhosis were similar qualitatively to those of the patients with more severe disease (Table I, Fig. 1). None of the changes were as severe. Three patients had MD within the normal range. The frequency distribution curves were abnormal in 3 cases and normal in 3.

TABLE I. MEAN ERYTHROCYTE VALUES OF NORMAL SUBJECTS AND PATIENTS WITH LIVER DISEASE

GROUP	NO. OF CASES	MD (μ)	CASES WITH CELLS >9.25 μ	S.D. OF CURVES	MCV (CU. μ)	MT (μ)	RBC (M)	HGB. (GM.)	MCHC (%)	RETICS. (%)
Normal*	16	7.77 ±0.29†	1‡	0.47 ±0.03	93 ±6.0	1.91 ±0.12	5.11 ±0.40	14.5 ±0.82	31 ±1.1	1.5 ±0.6
All cirrhosis	24	8.63 ±0.50	22	0.57 ±0.07	103 ±6.9	1.75 ±0.34	4.07 ±0.74	12.6 ±1.3	31 ±1.3	4.4 ±3.1
Significant cirrhosis	18	8.74 ±0.45	17	0.57 ±0.07	105 ±4.4	1.74 ±0.17	3.86 ±0.50	12.4 ±1.4	31 ±0.97	5.3 ±3.0
Mild cirrhosis	6	8.31	5	0.56	97	1.79	4.70	13.5	31	1.7
Alcoholic without cirrhosis	5	7.96	3	0.53	97	1.96	4.66	13.8	31	3.7
Hepatitis	3	8.71	3	0.60	95	1.59	4.60	14.3	33	4.7
Tumor	3	8.24	2	0.58	92	1.73	4.23	11.3	30	2.5

$$\text{MT (Mean thickness)} = \frac{\text{MCV}}{\frac{\pi\ (\text{MD})^2}{4}}$$

MCV—Mean corpuscular volume.
MCHC—Mean corpuscular hemoglobin concentration.
*RBC, Hgb., Hct., and reticulocytes determined for 11 of 16 normals.
†Standard deviation.
‡99.84 per cent of 3,200 normal cells had diameter between 6.25 to 9.25 μ inclusive.

Alcoholic.—The patients in this group were as severe and long-standing alcoholics as the patients with cirrhosis. None had any clinical or laboratory evidence of liver disease. Only one had an elevated MD, and only in this case was the frequency distribution curve of the type found in patients with cirrhosis.

The 29 patients who had a high alcoholic intake were grouped according to the degree of liver disease and, as demonstrated, there were differences in mean hematological values among these groups. However, as indicated in Table I and Fig. 1, there were no sharp lines separating the individual values of the patients of one group from those of another, but there was considerable overlapping.

Hepatitis.—All of the patients in this group had severe liver disease and in two cases the disease was of several months' duration. Like the group with cirrhosis, these patients had macrocytosis, an elevated average reticulocyte count, and the same type of abnormal frequency distribution curves. The group with hepatitis differed from the group with cirrhosis by a lower average MCV, a higher MCHC, a higher RBC, and a lower MT (Table I).

Tumor.—The patient with a hepatoma had a normal MD, 7.70 μ. The frequency distribution curve was skewed, but in contrast to the curves of patients with cirrhosis, microcytes and not macrocytes produced the abnormality of the curve. The two patients with metastatic tumor had an increased MD, and while the frequency distribution curves were shifted to the right, they were of normal configuration. All 3 patients had huge livers, but the function, as measured by the usual tests, was only slightly abnormal.

The Relationship Between Blood Values and the Course of the Disease.—One patient with hepatitis made a complete recovery and another recovered functionally although probably not morphologically. The majority of the patients with cirrhosis and one patient with hepatitis improved somewhat, but had residual liver damage at the time of maximal improvement. Almost all patients that improved showed a similar course. With improvement in hepatic function, the MD, MCV, and reticulocyte count tended to fall, and the RBC tended to rise. All values then leveled off, usually short of normal. This course is illustrated in Fig. 3, which was constructed from the data of Patient R. D., who was very ill with cirrhosis but made a striking improvement over a long hospitalization. The course of Patient H. L. with recurrent hepatitis is illustrated in Fig. 4. This patient got worse during the first three months of hospitalization. During this period the MD, MCV, and reticulocyte count rose. As the hepatitis began to improve, these trends in erythrocyte values reversed.

Four patients were given a combination of vitamin B_{12}, folic acid, and crude liver extract. Eight others received crude liver extract only, and one received vitamin B_{12} and crude liver extract. These supplements were given for at least one month and were started after the patient had shown the usual initial improvement and had become stabilized. In no instance was there any change in red cell size that could be attributed to the dietary supplements.

The Relationship Between Anemia and Cell Size.—There was a rough inverse correlation between the red cell count and the cell size. Cases with high mean diameters tended to have the lowest red cell counts. There was a total of 58 determinations of MD with simultaneous red blood counts. Twenty measurements of 8.35 μ and greater had an accompanying RBC of at least 4.0 million. Twelve of the group had an RBC of at least 4.5 M, and in 8, the RBC was at least 4.7 M. Thus, macrocytosis was frequently observed in the presence of a normal red count.

The Relationship Between Liver Function and Cell Size.—In general, the patients with cirrhosis and hepatitis who had the most disturbed liver function had the most marked erythrocyte abnormalities. Correlation between each quantitative liver function test and the initial MD determination of each patient with hepatitis or cirrhosis was attempted. The relationships between the MD

and the prothrombin time, serum albumin, alkaline phosphatase, and bromsulfalein (BSP) retention were roughly of a straight-line nature. The thymol turbidity, and total serum bilirubin and MD showed no such relationship. The

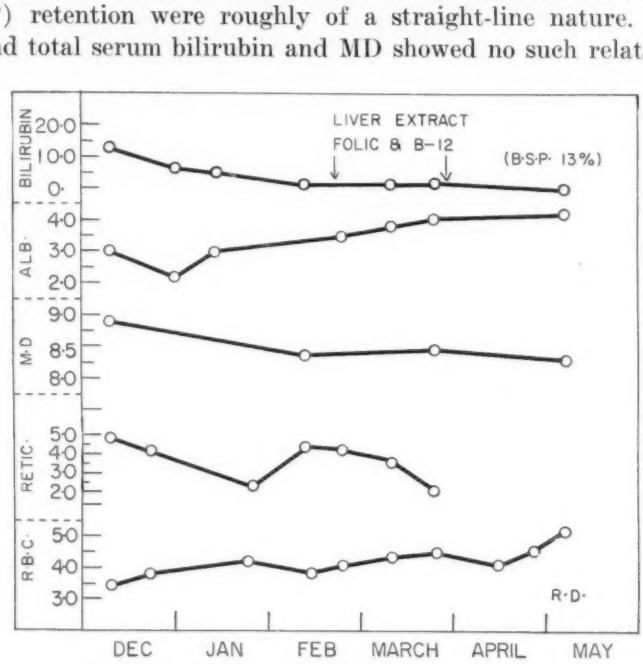

Fig. 3.—The course of the liver function tests and blood values of Patient R. D. with cirrhosis, who improved but did not recover completely.

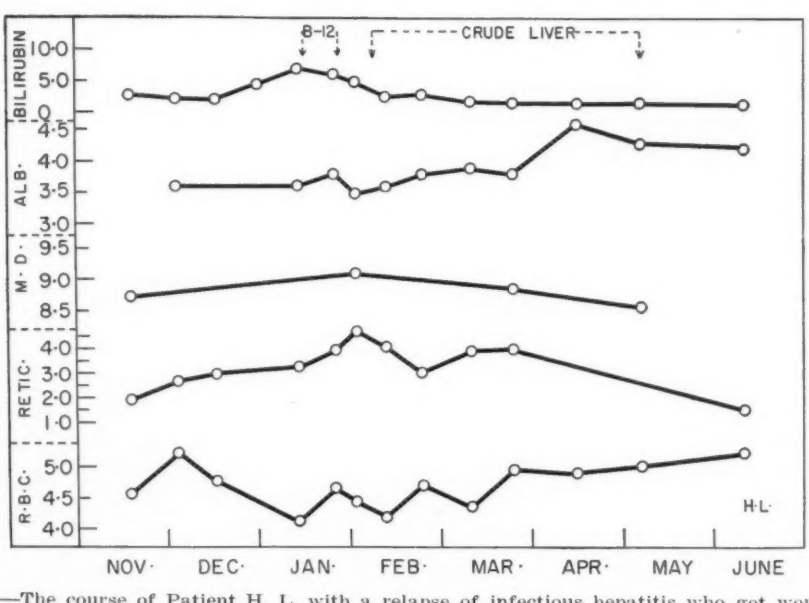

Fig. 4.—The course of Patient H. L. with a relapse of infectious hepatitis who got worse during his hospitalization and then improved but did not recover completely.

coefficients of correlation between each of the fairly well-correlated function tests and the MD were: prothrombin time (Owren) +0.49, albumin −0.48,

alkaline phosphatase +0.39, and BSP +0.16. Since the standard errors of these determinations were 0.20 to 0.22, the degree of correlation and the differences between the coefficients were not great in any case. The relationship between one of the best correlated function tests, the prothrombin time, and the MD is illustrated in Fig. 5.

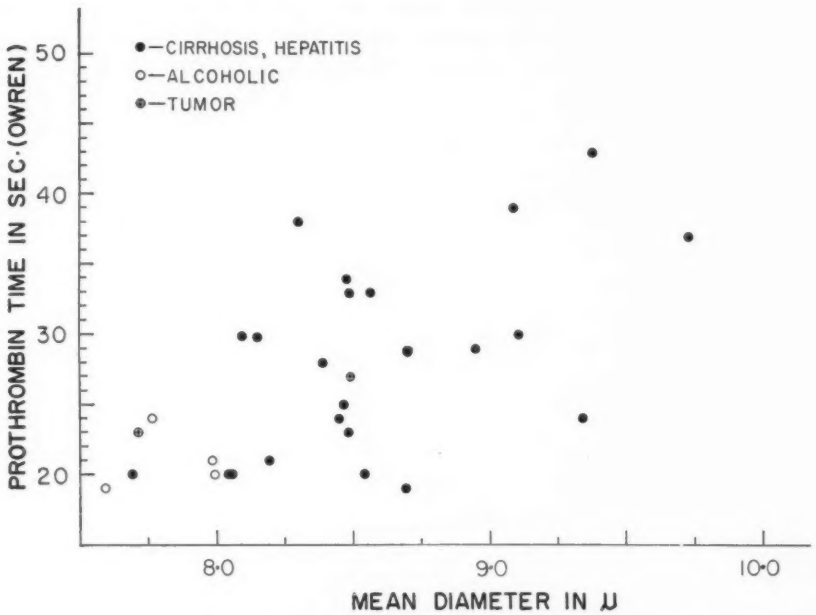

Fig. 5.—Relationship between MD and prothrombin time. Initial values for each patient with hepatic damage.

Analysis of the Red Cell Frequency Distribution Curves.—Larsen[8] analyzed his frequency distribution curves by a technique developed from a method described by Mogensen.[10] Larsen's method started with the assumption that the curves of patients with hepatitis could be divided into two cell populations, each having a normal distribution about its own mean. The differences between the two means varied between 0.75 to 1.0 μ. On this basis he constructed a series of theoretical curves with each curve representing a different proportion between the two cell populations, 90 to 10 per cent, 85 to 15 per cent, etc. By finding which of these theoretical curves best fitted the curve being analyzed, he determined the mean diameter of each component and the proportion of the components. This rough determination was checked by statistical methods. In the present study the analysis was not carried beyond the stage of making the rough determination of the mean diameter and of the number of cells of each of the two components. Almost all of the 47 abnormal curves could be broken down into two normally distributed cell populations. An example of curve analysis is given in Fig. 6. The MD of the total 200 cells measured in this case was 9.11 μ, and the frequency distribution curve was skewed. According to the analysis, 30 per cent of the cells was from a cell population normally distributed around a mean of 8.60 μ, and 70 per cent was from a population with a mean of

9.35 μ. The shape of the curves of total cell population in each patient depended on the relative sizes of the two components and whether the smaller component was on the right or the left of the larger. When the smaller component was on the right, the curve was skewed to the right. The latter configuration was the most common, as illustrated by the curve of Patient R. D. with cirrhosis, in Fig. 2. The differences between the means of the components varied between 0.75 and 1.0 μ. In some of the cases, the smaller of the two cell components had a normal MD, but in many cases both components had an increased MD.

Eight frequency curves were of normal configuration, but the MD was elevated. All cells showed an increase in size in a uniform manner. Three of these curves were initial curves of patients with significant or mild cirrhosis, 3 were from patients whose initial curve was of 2 components, and 2 were from the 2 patients with metastatic tumor.

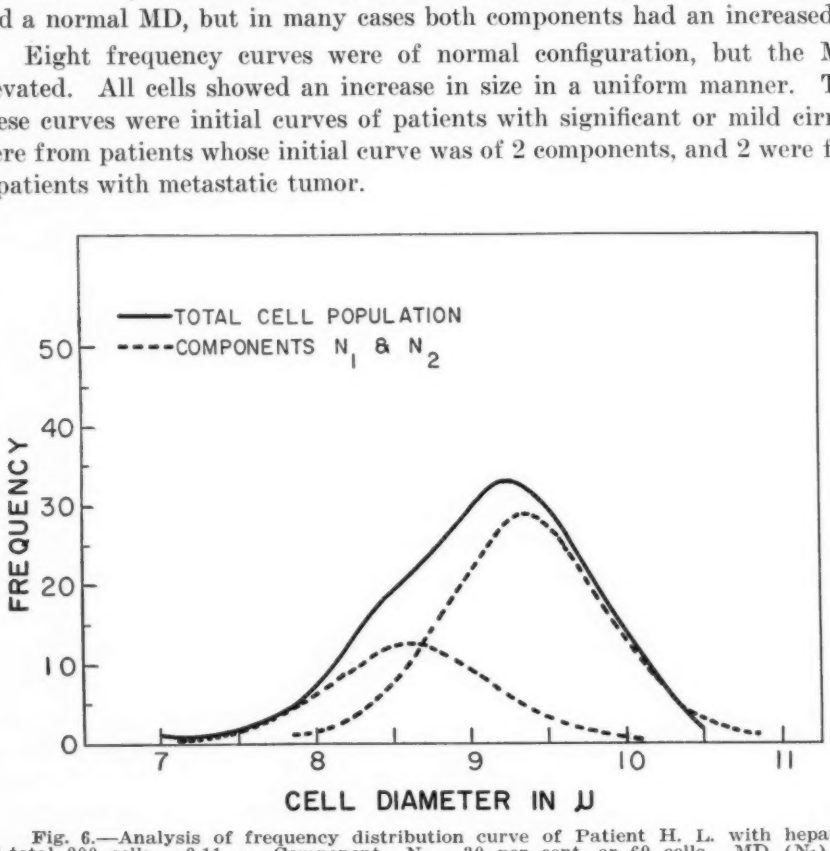

Fig. 6.—Analysis of frequency distribution curve of Patient H. L. with hepatitis. MD of total 200 cells – 9.11 μ. Component N_1 – 30 per cent, or 60 cells. MD (N_1) – 8.60 μ. Component N_2 – 70 per cent, or 140 cells. MD (N_2) – 9.35 μ.

DISCUSSION

The present study is not concerned strictly with the anemia associated with liver disease but with both the morphological and numerical changes in the erythrocytes. A high incidence of macrocytosis was found. This was higher than reported in several of the previous studies, because cell diameter was measured by a sensitive method of individual cell measurement, and because complications such as hemorrhage which would cause erythrocyte size changes independently of the liver disease were absent.

Larsen[8] found several abnormalities of erythrocyte size to be characteristic of the majority of his patients with hepatitis. Macrocytes, or cells larger than

the largest cells in normal erythrocyte populations, were present, but microcytes were not. While the macrocytes had greater diameters than normal cells, they were thin cells, and mean cell volume was not increased. The erythrocyte diameter frequency distribution curves were bicomponent with the mean diameter of one component in the normal range and the mean diameter of the right-hand component 0.75 to 1.0 μ greater. With clearing of the liver damage, the erythrocyte changes also disappeared. The present study demonstrated the same erythrocyte changes to be characteristic of the majority of a group of patients with Laennec's cirrhosis, with two probably important exceptions. Both components of the curve had a high mean diameter in some cases, and some patients had a high mean corpuscular volume. The increase in MCV was not as pronounced as the increase in MD. The data can best be interpreted to support the following theory: There is a type of macrocytosis that is common to both infectious hepatitis and Laennec's cirrhosis—the production of large, thin cells in addition to the normal cells. The factor producing this change is probably effective during erythropoiesis. In cirrhosis and perhaps in some cases of chronic hepatitis, an additional factor or factors tend to increase over-all cell size. These factors appear to act uniformly on all cells and could be effective either during erythropoiesis or after the cells have left the bone marrow.

The macrocytosis of liver disease is unlike that of pernicious anemia in relapse.[4, 8, 10, 20, 21] The erythrocyte frequency distribution curves of liver disease are like those of pernicious anemia in partial remission, and situations where macrocytosis persists after an otherwise complete remission of pernicious anemia.[20-25] This similarity is of interest and of possible etiological significance.

The cause or causes of the erythrocyte abnormalities of liver disease are unknown. Larsen[8] summarized the possible mechanisms, and there is no need to review them here. Liver damage is the one obvious factor common to both hepatitis and cirrhosis. The degree of macrocytosis is roughly correlated with the degree of liver dysfunction. Since viral infection, inadequate diet, or high alcohol intake are not all common to both hepatitis and cirrhosis, none of these factors can be the basic cause of the macrocytosis. This conclusion does not exclude the possibility that one or all of these factors have some effect on erythrocyte production or destruction.

Frank hemolytic anemia or an increased rate of erythrocyte destruction may accompany liver disease.[16, 17, 26-28] Increased erythropoiesis and reticulocytosis normally follow increased erythrocyte destruction, and reticulocytosis has been observed in several studies including the present study. An increased number of reticulocytes would then seem to be a possible cause of the macrocytosis. However, Persons[29] found that normal reticulocytes are about 1.0 μ larger than normal adult erythrocytes, and it is unlikely that the average 0.97 μ increase in MD of our patients with significant cirrhosis could have been caused by the average reticulocytosis of only 5.3 per cent.

SUMMARY AND CONCLUSIONS

Chronic or severe acute liver damage of several types is usually accompanied by an increase in the mean diameter of erythrocytes and by the presence of

abnormally large erythrocytes. The individual erythrocytes are not distributed about a mean diameter in a normal manner, but in most cases there appear to be two components of the cell population each normally distributed about its mean. The macrocytosis often is present in the absence of anemia. The degree of macrocytosis can be roughly correlated with the degree of abnormality of liver function. With improvement in liver function, there is usually a fall in the mean diameter toward normal, but it is likely to remain elevated as long as liver damage is present.

While the cause of the macrocytosis appears to be liver damage, the mechanism is unknown. The macrocytosis is not due solely to the presence of an increased number of reticulocytes, to dietary deficiencies, to a high alcoholic intake, or to a simple swelling of the erythrocytes, although these may be contributing factors.

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THE NUCLEIC ACIDS AND OTHER PHOSPHORUS COMPOUNDS OF HUMAN LEUKEMIC LEUKOCYTES: RELATION TO CELL MATURITY

DEMETRIOS A. RIGAS, PH.D., MARIE L. DUERST, B.A., MARGARET E. JUMP, PH.D., AND EDWIN E. OSGOOD, M.D.
PORTLAND, ORE.

THE phosphorus compounds of the cells, which include such important substances as adenosine triphosphate (ATP), ribonucleic acid (RNA), and desoxyribonucleic acid (DNA), undoubtedly play a vital role in the processes of cell growth, division, and differentiation. Knowledge concerning the cellular content, rate of metabolism, and relationship of these compounds to cell growth, maturity, or malignancy will contribute substantially to a better understanding of these processes. However, the direct chemical analysis of different tissues usually suffers from certain serious limitations. The presence of blood vessels, connective tissue, etc., introduces an error of unknown magnitude. The difficulty encountered in estimation of the number of cells and percentages of different types of cells is another serious handicap. In contrast, leukocytes are exceptionally suitable for such studies, since they can be easily obtained as discrete, undamaged cells in a pure state. The total number of leukocytes, the per cent of each type, and the per cent at each stage of maturity can be accurately determined.

In a preliminary report,[1] we summarized the results of a large number of chemical analyses of the phosphorus compounds of human leukemic leukocytes. It is the purpose of this paper to present these studies in detail and to discuss the relationship of these compounds to the type, size, and maturity of the leukocytes.

MATERIALS AND METHODS

The leukocytes used in these studies were obtained from 62 patients with various types of leukemic leukemias as shown in Tables II, III, and IV. The leukocyte counts of these patients ranged from 50,000 to 500,000 per cubic millimeter of blood. Whenever possible the leukocytes of each patient were analyzed on several different occasions, usually more than three. The first blood specimen was usually obtained before P^{32} therapy was started.[2] Subsequent samples were drawn at intervals within the first two weeks after initiation of treatment with P^{32}. The amount of blood necessary to yield at least 0.5 ml. of packed leukocytes, calculated from the patient's leukocyte count and average leukocyte size,[3] was obtained by venipuncture, with use of heparin as the anticoagulant. The blood was immediately chilled in ice, and processed as quickly as possible.

From the Department of Medicine, Division of Experimental Medicine, University of Oregon Medical School, Portland, Ore.

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Separation of the Leukocytes.—Prompt separation of the leukocytes from erythrocytes was attained by the phytohemagglutinin technique[4, 5] at about 4° C. Duplicate leukocyte counts were performed on the final suspension in their own plasma. Differential counts of at least 500 cells were made on Wright's stained smears prepared from this suspension.* Differential counts obtained from such smears were in good agreement with those obtained from direct blood smears. On the average, 96 per cent of the leukocytes from patients with chronic granulocytic leukemia were of the granulocytic series, and 99 per cent of those from patients with lymphocytic leukemia were of the lymphocytic series. The suspension of leukocytes was centrifuged in a calibrated Osgood tube† at about 4° C. and 2,700 g until a constant leukocyte volume was obtained. The total number and the size of the leukocytes were calculated as described in an earlier paper.[3] Following removal of the supernatant plasma, the packed leukocytes were used without washing, since a considerable loss of leukocyte phosphorus was observed after washing,‡ and the amount of trapped plasma in packed leukocytes appears to be negligible.[3]

Fractionation Methods.—The Schmidt and Thannhauser method[7] was chosen for these studies because it offers the possibility of studying P^{32} uptake into DNA, which can be used as a measure of the mitotic rate and lifespan of the leukocytes.[8-10] In the early phase of this work, desoxyribonucleic acid phosphorus (DNA-P) and total phosphorus were the only fractions determined, with use of a simplified Schmidt and Thannhauser method specially developed for this purpose. Later the complete Schmidt and Thannhauser method,[7] slightly modified, was used for fractionation into inorganic phosphorus, acid soluble organic phosphorus, phospholipid phosphorus, ribonucleic acid phosphorus (RNA-P), desoxyribonucleic acid phosphorus (DNA-P), and phosphoprotein phosphorus fractions.

Simplified Schmidt and Thannhauser Method.—The packed leukocytes were suspended in 40 volumes of 1 N KOH and incubated for 36 hours at 37° C. The clear digest was brought to room temperature, and an aliquot was used for total phosphorus determination. DNA was determined on a separate aliquot as originally described by Schmidt and Thannhauser.[7] Phosphorus determinations were made by the Fiske and Subbarow method,[11] following wet digestion. Evidence that the DNA-P fraction was not contaminated by phosphorus from other fractions was obtained as follows.

Highly polymerized desoxyribonucleoprotein (DNP) was prepared by the Mirsky and Pollister method[12] from the leukocytes of a patient with chronic granulocytic leukemia. Quadruplicate analyses for DNA-P were then performed by the simplified Schmidt and Thannhauser procedure on 5.0 ml. aliquots of DNP solution, either alone or after addition of: (a) 100 mg. of dried egg powder (containing 0.76 per cent P, mostly phospholipids and phosphoproteins), (b) 5.0 ml. of human erythrocytes, (c) 5.0 ml. of human plasma, or (d) 0.2 ml. of human leukemic lymphocytes. Increase in the DNA-P of the DNP solution was produced only by the addition of lymphocytes, as shown in Table I. A comparison of the simplified and complete Schmidt and Thannhauser methods by triplicate analyses on leukocytes of three patients with different types of leukemia (Table I) demonstrated good agreement between the two methods.

Complete Schmidt and Thannhauser Method.—The following slight modifications of the original method[7] were made: The leukocytes were homogenized with a glass homogenizer§ in

*The terms and definitions recommended by the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs[6] were followed.

†These tubes are available from Arthur H. Thomas Co., Philadelphia, Pa., Catalog No. 3151.

‡When 1.0 ml. of leukocytes from a patient with chronic granulocytic leukemia was washed three times with isotonic saline, 0.150 mg. of inorganic and 0.050 mg. of acid soluble organic phosphorus were removed by this process. The plasma of this patient contained only 0.026 mg. of inorganic and 0.043 mg. of acid soluble organic phosphorus per milliliter, which indicates that even if most of the volume of leukocytes consisted of trapped plasma, the latter would not explain the loss of phosphorus by washing.

§Available from Microchemical Specialties Co., 1834 University Avenue, Berkeley 3, Calif., Catalog No. 7510.

TABLE I. COMPARISON OF THE SIMPLIFIED SCHMIDT AND THANNHAUSER METHOD WITH THE COMPLETE METHOD

MATERIAL ANALYZED	NUMBER OF DETERMINATIONS	DNA-P	
		SIMPLIFIED METHOD	COMPLETE METHOD
		Milligrams	
5.0 ml. of DNP solution	4	2.85	
5.0 ml. of DNP plus 100 mg. egg powder	4	2.73	
5.0 ml. of DNP plus 5.0 ml. human erythrocytes	4	2.85	
5.0 ml. of DNP plus 5.0 ml. human plasma	4	2.84	
5.0 ml. of DNP plus 0.2 ml. human lymphocytes	4	3.35	
		Mg. Per 10^9 Leukocytes	
Leukocytes from chronic granulocytic leukemia	3	0.670	0.667
Leukocytes from chronic lymphocytic leukemia	3	0.591	0.594
Leukocytes from acute monocytic leukemia	3	0.633	0.625

10 times their volume of 5 per cent trichloroacetic acid, followed by addition of 15 volumes of 10 per cent trichloroacetic acid, thus raising its concentration to 8 per cent. The clear supernatant containing the acid soluble phosphorus compounds was obtained by centrifugation. An aliquot of this supernatant was used for inorganic phosphorus determination by the Delory method,[13] while total acid soluble phosphorus was determined in another aliquot. All work up to precipitation of the inorganic phosphorus was performed at about 1° C. Extraction of the phospholipids from the 8 per cent trichloroacetic acid precipitate was carried out immediately by three aliquots of Bloor's solution (3 volumes ethanol plus 1 volume ethyl ether), each about 10 to 25 times the volume of the original leukocytes, followed by two equal aliquots of ethyl ether. The extraction was performed near the boiling point of the extracting liquid, keeping the residue well dispersed. Further extraction with chloroform was found to be unnecessary. The extracted residue was subsequently digested for 36 hours at 37° C. in 30 times the original volume of the leukocytes of 1 N KOH and processed as originally described by Schmidt and Thannhauser.[7] All precipitates were recovered by centrifugation of sufficient speed and duration to clarify the supernatant liquid and were repeatedly washed with solution of a composition similar to the supernatant. Following wet digestion, phosphorus determinations on each fraction were carried out by the Fiske and Subbarow method.[11]

Computation.—Duplicate phosphorus determinations were performed on each fraction and the mean used to calculate the content per billion cells or per milliliter of cells. Acid-soluble organic phosphorus was obtained by subtracting the inorganic from the total acid-soluble phosphorus. Similarly, the RNA-P was calculated as the difference between the phosphoprotein-P and the non DNA-P of the KOH digest. The rest of the fractions were obtained directly, with the exception of the total phosphorus in the complete Schmidt and Thannhauser method, where it was calculated as the sum of the total acid soluble-P, phospholipid-P, and KOH-digest total phosphorus. The error in these values due to contamination of the leukocytes by erythrocytes and thrombocytes was disregarded, as the maximum contamination was about 1 per cent and 3 per cent, respectively, by volume, leading to a 3 per cent maximum error in the phosphorus values.

Statistical Methods.—The distributions of the data were tested for skewness and kurtosis[14] and accepted as normal at the 5 per cent level of significance. The normally distributed data were then tested for homogeneity of variance according to Box,[15] and the means of the groups were compared by analysis of variance, Student's "t" test, or by the Behrens-Fisher method,[16] using Sukhatme's tables[17] when the variances were unequal. All these tests were performed at the 5 per cent level of significance. Groups of data on a particular phosphorus fraction from different patients or different leukemias showing nonsignificant

differences were pooled together and their mean ($\overline{\overline{X}}$) and standard deviation ($\overline{\overline{s}}$) were calculated with use of the equations:

$$\overline{\overline{X}} = \frac{\Sigma\, x_{ij}}{\Sigma\, n_i} \quad \text{and} \quad \overline{\overline{s}} = \sqrt{\frac{\Sigma\, x_{ij}^2 - \frac{(\Sigma\, x_{ij})^2}{\Sigma\, n_i}}{\Sigma\, n_i - 1}}$$

where x_{ij} is the jth determination on the ith group, and n_i the number of determinations on the ith group. Histograms and lines on arithmetic probability paper were drawn for such data by the usual techniques. Whenever statistically significant differences were observed between the means of different patients, a weighted mean ($\tilde{\overline{X}}$) and weighted standard deviation ($\tilde{\overline{s}}$) for the group of patients were calculated by the equations:

$$\tilde{\overline{X}} = \frac{\Sigma\, (\overline{X}_i\, \sqrt{n_i})}{\Sigma\, \sqrt{n_i}} \quad \text{and} \quad \tilde{\overline{s}} = \sqrt{\frac{\Sigma\, [(\overline{X}_i - \tilde{\overline{X}})^2\, \sqrt{n_i}]}{\Sigma\, \sqrt{n_i} - 1}}$$

where $\overline{X}_i$ is the mean of n_i determinations on the ith patient. Each mean $\overline{X}_i$ was assigned a frequency equal to $\sqrt{n_i}$. With use of the sum of these frequencies, rather than the number of such means falling into a particular arbitrary range, histograms and lines on arithmetic probability paper were drawn.

Groups showing significant differences were subjected to multiple regression analysis[16] in which the percentages of the cells of different differentiation stages and size of cells were taken as the independent variables. The resulting multiple regression coefficient and the standard partial regression coefficients were tested for significance at the 1 per cent level by the F and "t" tests, respectively. Variables leading to nonsignificant regression coefficients were dropped and the regression equation for the remaining variables was derived. Ninety-five per cent confidence limits for the regression coefficients and the phosphorus values derived from the equations were calculated.

RESULTS

Amount of Phosphorus Compounds Per Billion Human Leukemic Leukocytes.—The results obtained from 235 analyses of leukocytes from the blood of 62 patients with different types of leukemic leukemias are tabulated as milligrams of P per 10^9 cells in Tables II, III, and IV. Data on individual patients, where a relatively large number of determinations were performed, suggest that they can be considered normally distributed ($P > 0.05$). We have assumed that this is true in every patient. The variances of the phosphorus fractions were found to be homogeneous at the 5 per cent level of significance among patients with the same type of leukemia.

Data obtained by the simplified Schmidt and Thannhauser technique (Table II) when compared by the "t" test to those obtained by the complete method (Tables III and IV) were not found to be significantly different. The DNA-P, as determined by the simplified method, was found not different from that obtained by the complete method ($P \sim 0.40$). The same is true of the total-P in chronic granulocytic leukemias ($P \sim 0.32$), as well as in the chronic lymphocytic leukemias ($P \sim 0.10$). The possible effect of the P^{32} therapy on each fraction was similarly tested by comparing data obtained prior to the initiation of therapy to those obtained after P^{32} therapy was started. No

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TABLE II. RESULTS OBTAINED BY THE SIMPLIFIED SCHMIDT AND THANNHAUSER METHOD ON THE DNA-P AND TOTAL-P OF HUMAN BLOOD LEUKOCYTES IN DIFFERENT TYPES OF LEUKEMIA

PATIENT	DNA-P (MG./10^9 CELLS)	TOTAL-P (MG./10^9 CELLS)	SIZE OF CELLS (ML./10^9 CELLS)	PATIENT	DNA-P (MG./10^9 CELLS)	TOTAL-P (MG./10^9 CELLS)	SIZE OF CELLS (ML./10^9 CELLS)	PATIENT	DNA-P (MG./10^9 CELLS)	TOTAL-P (MG./10^9 CELLS	SIZE OF CELLS (ML./10^9 CELLS)
					Chronic Granulocytic Leukemia						
R. G.	0.520	1.452	0.406	B. D.	0.743	1.783	0.569	A. M.	0.538	1.227	0.417
R. G.	0.602	1.594	0.412	B. D.	0.579	1.485	0.455	A. M.	0.552	1.616	0.410
N. E.	0.735	1.825	0.472	B. D.	0.478	1.524	0.519	A. M.	0.563	1.684	0.430
N. E.	0.730	1.739	0.501	B. E.	0.726*	1.894*	0.549	A. M.	0.518	1.634	0.403
N. E.	0.661	1.562	0.490	B. E.	0.708	1.909	0.607	A. M.	0.614	1.874	0.570
N. E.	0.581	1.408	0.414	B. E.	0.689	1.795	0.556	A. M.	0.702	1.686	0.495
N. E.	0.632	1.819	0.360	B. E.	0.711	1.788	0.522	A. M.	0.709	1.674	0.468
M. L.	0.722*	1.847*		B. E.	0.590	1.561	0.483	K. W.	0.623	1.465	0.583
M. L.	0.555	1.408		B. E.	0.551	1.538	0.441	K. W.	0.488	1.473	0.424
M. L.	0.528	1.250	0.422	A. M.	0.510*			O. I.	0.594*	1.421*	0.644
M. L.	0.543	1.425	0.464	A. M.	0.307			O. I.	0.723	1.570	0.489
M. L.	0.638	1.521	0.462	A. M.	0.602			O. I.	0.584	1.769	
M. L.	0.633	1.641	0.458	A. M.	0.632			D. M.	0.398*	1.679*	0.520
M. L.	0.574	1.490	0.446	A. M.	0.439			D. M.	0.842	1.784	0.617
M. L.	0.694	1.738	0.420	A. M.	0.855			D. M.	0.633	1.729	0.617
M. L.	0.585	1.661	0.465	F. N.	0.672*	1.680*	0.437	M. O.	0.735	1.847	0.493
M. L.	0.631	1.592	0.457	F. N.	0.580	1.456	0.392	M. O.	0.504	1.457	0.431
M. L.	0.690	1.672	0.464	F. N.	0.424	1.251	0.349	M. O.	0.732	1.914	0.565
M. L.	0.817	2.112	0.460	F. N.	0.566	1.723	0.459	W. E.	0.818*		
B. D.	0.659*	1.730*	0.495	A. M.	0.845*			W. E.	0.784		
B. D.	0.565	1.445	0.442	A. M.	0.467*	1.046*	0.566				
					Chronic Lymphocytic Leukemia						
J. J.	0.511	1.010	0.349	K. P.	0.646	1.747	0.477	D. A.	0.449	0.822	0.191
T. M.	0.691	1.590	0.362	S. S.	0.612	1.056	0.175	B. G.	0.671*	1.277*	0.272
H. L.	0.618	1.069	0.190	S. S.	0.572	1.076	0.211	B. G.	0.576*	1.073*	0.232
H. L.	0.563	0.964	0.163	S. S.	0.580	1.104	0.203	B. G.	0.532	1.067	0.356
H. L.	0.543	0.865	0.156	S. S.	0.595	1.193	0.248	B. G.	0.687	1.387	0.197
H. L.	0.606	0.993	0.169	Mc. A.	0.702	1.256	0.260	B. G.	0.593	1.151	0.226
H. L.	0.596	1.016	0.182	Mc. A.	0.641	1.198	0.224	B. G.	0.588	1.124	0.198
H. L.	0.614	1.053	0.179	Mc. A.	0.631	1.214	0.245	J. E.	0.505*	0.909*	0.154
H. L.	0.562	1.007	0.156	Mc. A.	0.593	1.106	0.234	J. E.	0.524*	0.939*	0.162
H. L.	0.542	0.939	0.150	R. O.	0.605*	1.137*	0.205	J. E.	0.506	0.940	0.162
H. L.	0.574	0.991	0.180	W. W.	0.595*	0.904*	0.158	J. E.	0.616	1.132	0.208
F. N.	0.558*	0.957*	0.153	W. W.	0.562	0.854	0.220	J. E.	0.675	1.229	0.234

F. N.	0.610	1.046	0.183	W. W.	0.535	0.847	0.165	J. E.	0.579	1.086	0.229
F. N.	0.570	0.969	0.167	W. W.	0.538	1.016	0.228	H. A.	0.529*	1.069*	0.311
F. N.	0.631	1.025	0.192	G. H.	0.567	1.068	0.223	H. A.	0.570	1.185	0.352
F. N.	0.603	1.053	0.208	G. H.	0.568	1.051	0.125	H. A.	0.412	0.858	0.242
K. P.	0.683*	1.519*	0.282	G. H.	0.623	1.189	0.242	H. A.	0.437	1.061	0.248
K. P.	0.638	1.715	0.393	D. A.	0.649	1.172	0.268				
					Acute Lymphocytic Leukemia						
M. S.	0.521*	1.056*	0.248	M. S.	0.620	1.277	0.360	C. D.	0.561*	1.351*	0.326
M. S.	0.603	1.259	0.296	M. S.	0.650	1.419	0.332	C. D.	0.508	1.145	0.309
M. S.	0.585	1.276	0.291	M. S.	0.621	1.331	0.372				
					Subacute Monocytic Leukemia						
Y. A.	0.569*	1.483*	0.391	Y. A.	0.703	1.818	0.545	H. T.	0.731	1.972	0.584
Y. A.	0.668	1.746	0.508	Y. A.	0.641						
					Acute Monocytic Leukemia						
B. L.	0.567*	1.363*	0.340	C. W.	0.617*	1.781*	0.446	C. W.	0.654	2.200	0.550
B. L.	0.660*	1.617*	0.399	C. W.	0.688	2.083	0.538	C. W.	0.569	2.011	0.584
C. W.	0.609*	1.802*	0.430	C. W.	0.709	2.378	0.648	C. W.	0.749	2.596	0.510

*These determinations were made prior to treatment with P^{32}.

TABLE III. RESULTS OBTAINED BY THE COMPLETE SCHMIDT AND THANNHAUSER METHOD ON THE PHOSPHORUS COMPOUNDS OF HUMAN BLOOD LEUKOCYTES IN GRANULOCYTIC AND MONOCYTIC LEUKEMIAS

PATIENT	INORGANIC-P (MG./10^9 CELLS)	ACID SOLUBLE ORGANIC-P (MG./10^9 CELLS)	PHOSPHOLIPID-P (MG./10^9 CELLS)	RNA-P (MG./10^9 CELLS)	PHOSPHOPROTEIN-P (MG./10^9 CELLS)	DNA-P (MG./10^9 CELLS)	TOTAL-P (MG./10^9 CELLS)	CELLS WITH NUCLEOLI (%)	SEGMENTED CELLS (%)	CELL SIZE (ML./10^9 CELLS)
					Chronic Granulocytic Leukemia					
D. M.	0.077*	0.309*	0.352*	0.214*	0.017*	0.599*	1.546*	6.6	30.2	0.586
J. A.	0.124*	0.275*	0.343*	0.288*	0.022*	0.585*	1.619*	3.0	19.0	0.702
R. A.	0.129*	0.419*	0.413*	0.410*	0.027*	0.701*	2.057*	18.2	12.0	0.539
H. M.	0.119*	0.274*	0.317*	0.419*	0.024*	0.441*	1.576*	11.0	11.0	0.548
Mc. D.	0.115	0.328	0.362	0.319	0.026	0.704	1.851	5.2	9.8	0.616
Mc. D.	0.061	0.228	0.275	0.203	0.013	0.459	1.246	2.0	16.0	0.632
Mc. D.	0.046	0.219	0.258	0.179	0.010	0.460	1.166	2.6	15.2	0.694
W. E.	0.113	0.372	0.379	0.331	0.027	0.639	1.836	14.4	17.6	0.664
W. E.	0.096	0.448	0.407	0.388	0.032	0.637	2.002	24.8	21.6	0.632
W. E.	0.110	0.428	0.411	0.383	0.028	0.695	2.009	24.0	23.2	0.694
M. O.	0.088	0.445	0.435	0.347	0.027	0.676	2.013	16.6	7.8	0.866
M. O.	0.109	0.476	0.385	0.450	0.030	0.672	2.132	35.0	4.4	0.689
E. M.	0.060*	0.502*	0.527*	0.264*	0.019*	0.562*	1.935*	3.2	44.4	0.762
E. M.	0.082	0.390	0.491	0.204	0.017	0.570	1.731	3.1	40.1	0.710
E. M.	0.087	0.342	0.398	0.194	0.015	0.535	1.555	0.4	61.6	0.493
O. I.	0.093	0.357	0.375	0.316	0.026	0.569	1.773	21.8	8.2	0.498
O. I.	0.092	0.366	0.342	0.367	0.023	0.613	1.759	25.0	13.2	0.512
B. F.	0.051*	0.251*	0.301*	0.193*	0.014*	0.481*	1.290*	5.8	26.6	0.443
B. F.	0.073	0.342	0.399	0.259	0.019	0.601	1.721	4.2	22.4	0.543
B. F.	0.064	0.296	0.358	0.216	0.014	0.580	1.522	2.2	23.2	0.514
E. D.	0.083*	0.310*	0.365*	0.256*	0.018*	0.580*	1.614*	13.2	7.8	0.573

E. D.	0.078	0.286	0.332	0.213	0.016	0.519	1.441	14.4	17.2	0.427
E. D.	0.080	0.340	0.369	0.254	0.019	0.625	1.682	19.8	21.8	0.493
E. D.	0.098	0.362	0.425	0.316	0.020	0.664	1.879	16.4	15.6	0.544
E. D.	0.082	0.313	0.358	0.246	0.019	0.593	1.597	12.4	19.0	0.468
F. J.	0.071*	0.315*	0.358*	0.189*	0.017*	0.657*	1.616*	6.4	39.2	0.517
F. J.	0.086	0.261	0.353	0.176	0.016	0.619	1.522	11.0	35.6	0.475
F. J.	0.070	0.249	0.312	0.173	0.016	0.584	1.394	10.4	36.0	0.409
F. J.	0.097	0.279	0.365	0.195	0.022	0.654	1.640	9.2	31.6	0.519
F. J.	0.079	0.257	0.375	0.177	0.017	0.619	1.515	9.8	31.8	0.489
B. S.	0.101*	0.315*	0.384*	0.264*	0.018*	0.638*	1.714*	14.8	23.0	0.536
B. S.	0.107*	0.294*	0.393*	0.247*	0.027*	0.651*	1.735*	18.8	18.2	0.488
B. S.	0.078	0.260	0.310	0.233	0.021	0.535	1.456	10.6	29.8	0.425
B. S.	0.100	0.360	0.432	0.268	0.028	0.672	1.879	7.6	29.2	0.599
B. S.	0.094	0.325	0.411	0.263	0.027	0.650	1.751	10.6	17.8	0.509
S. M.	0.077	0.259	0.320	0.185	0.020	0.605	1.460	9.6	18.8	0.394
S. M.	0.072	0.276	0.315	0.193	0.022	0.583	1.462	9.4	18.2	0.375
				Chronic Eosinophilic Leukemia						
W. S.	0.071	0.184	0.556	0.197	0.020	0.687	1.750	0.0	63.2	0.547
W. S.	0.079	0.394	0.442	0.185	0.018	0.694	1.793	0.4	59.0	0.673
W. S.	0.067	0.335	0.369	0.179	0.017	0.514	1.498	0.2	72.4	0.479
W. S.	0.092	0.477	0.523	0.232	0.022	0.767	2.091	0.0	61.6	0.801
W. S.	0.094	0.471	0.517	0.254	0.019	0.700	1.990	0.0	63.4	0.845
				Acute Monocytic Leukemia						
T. W.	0.148*	0.408*	0.353*	0.440*	0.039*	0.871*	2.262*			0.544
T. W.	0.131	0.290	0.256	0.363	0.033	0.605	1.682			0.461
F. A.	0.053*	0.414*	0.325*	0.547*	0.036*	0.744*	2.025*	75.6		0.546

*These determinations were made prior to treatment with P^{32}.

TABLE IV. RESULTS OBTAINED BY THE COMPLETE SCHMIDT AND THANNHAUSER METHOD ON THE PHOSPHORUS COMPOUNDS OF HUMAN BLOOD LEUKOCYTES IN LYMPHOCYTIC LEUKEMIAS

PATIENT	INORGANIC-P (MG./10^9 CELLS)	ACID SOLUBLE ORGANIC-P (MG./10^9 CELLS)	PHOSPHOLIPID-P (MG./10^9 CELLS)	RNA-P (MG./10^9 CELLS)	PHOSPHOPROTEIN-P (MG./10^9 CELLS)	DNA-P (MG./10^9 CELLS)	TOTAL-P (MG./10^9 CELLS)	LYMPHOBLASTS AND PROLYMPHOCYTES (%)	CELL SIZE (MIL./10^9 CELLS)
				Chronic Lymphocytic Leukemia					
Mc. W.	0.066*	0.194*	0.133*	0.163*	0.017*	0.602*	1.171*	1.6	0.274
Mc. W.	0.066	0.235	0.151	0.201	0.017	0.709	1.374	3.8	0.317
Mc. W.	0.066	0.163	0.142	0.215	0.020	0.775	1.380	6.6	0.503
Mc. W.	0.040	0.177	0.116	0.164	0.013	0.510	0.998	10.0	0.237
Mc. W.	0.061	0.218	0.160	0.295	0.033	0.556	1.311	6.6	0.319
C. F.	0.063*	0.194*	0.156*	0.201*	0.020*	0.616*	1.238*	38.4	0.328
C. F.	0.042	0.254	0.175	0.238	0.021	0.647	1.357	34.4	0.368
C. F.	0.042	0.230	0.183	0.233	0.020	0.545	1.129	50.6	0.346
R. W.	0.085*	0.301*	0.203*	0.317*	0.031*	0.565*	1.510*	15.4	0.372
R. W.	0.050	0.249	0.148	0.230	0.022	0.447	1.157	15.6	0.263
R. W.	0.074	0.309	0.194	0.301	0.029	0.567	1.482	14.0	0.354
R. W.	0.075	0.327	0.210	0.304	0.032	0.594	1.565	17.0	0.370
R. W.	0.075	0.286	0.196	0.290	0.026	0.565	1.447	20.8	0.381
K. V.	0.065*	0.274*	0.208*	0.282*	0.018*	0.654*	1.485*	9.6	0.399
K. H.	0.029*	0.206*	0.138*	0.179*	0.014*	0.500*	1.077*	11.0	0.233
C. H.	0.028*	0.215*	0.163*	0.125*	0.011*	0.479*	1.024*	9.2	0.244
C. H.	0.037	0.255	0.191	0.171	0.015	0.583	1.244	9.0	0.244
C. H.	0.042	0.277	0.200	0.207	0.018	0.645	1.394	7.2	0.282
C. H.	0.041	0.188	0.161	0.143	0.014	0.570	1.141	9.4	0.299
C. H.	0.029	0.202	0.139	0.144	0.014	0.510	1.039	8.2	0.244
D. F.	0.020*	0.134*	0.091*	0.107*	0.021*	0.478*	0.831*	2.0	0.196
D. F.	0.024	0.160	0.111	0.096	0.010	0.548	0.939	6.8	0.199
D. F.	0.027	0.144	0.106	0.096	0.010	0.513	0.905	5.2	0.197
D. F.	0.033	0.148	0.110	0.098	0.010	0.545	0.938	5.4	0.203

M. L.	0.031*	0.237*	0.138*	0.166*	0.016*	0.542*	1.120*	14.0	0.265
M. L.	0.035	0.224	0.137	0.162	0.016	0.570	1.122	10.8	0.273
M. L.	0.032	0.219	0.132	0.150	0.015	0.537	1.085	10.4	0.245
M. L.	0.033	0.204	0.125	0.148	0.015	0.515	1.042	16.8	0.253
M. L.	0.028	0.210	0.125	0.153	0.016	0.506	1.044	16.8	0.244
C. E.	0.034*	0.162*	0.125*	0.143*	0.016*	0.525*	0.990*	1.4	0.232
C. E.	0.038	0.205	0.148	0.154	0.019	0.645	1.203	3.6	0.269
C. E.	0.033	0.176	0.129	0.136	0.012	0.562	1.055	1.8	0.211
C. E.	0.028	0.165	0.115	0.130	0.012	0.523	0.976	1.6	0.204
C. E.	0.033	0.197	0.151	0.159	0.015	0.582	1.130	2.8	0.210
H. D.	0.065*	0.153*	0.143*	0.148*	0.016*	0.575*	1.095*	7.8	0.248
H. D.	0.035	0.192	0.146	0.136	0.014	0.578	1.106	9.2	0.259
H. D.	0.029	0.205	0.155	0.152	0.016	0.600	1.146	15.2	0.252
H. D.	0.036	0.190	0.158	0.151	0.015	0.574	1.121	6.2	0.263
H. D.	0.039	0.207	0.160	0.174	0.018	0.621	1.228	10.4	0.290
H. J.	0.030*	0.125*	0.086*	0.096*	0.010*	0.511*	0.848*	9.4	0.174
H. J.	0.032	0.149	0.102	0.117	0.013	0.611	1.049	9.8	0.203
H. J.	0.028	0.124	0.087	0.097	0.011	0.511	0.854	9.6	0.172
H. J.	0.034	0.152	0.106	0.171	0.020	0.550	1.036	10.2	0.210
G. R.	0.038	0.343	0.153	0.181	0.017	0.651	1.400	10.8	0.297
G. R.	0.038	0.295	0.144	0.166	0.014	0.575	1.237	14.0	0.260
C. C.	0.041	0.191	0.129	0.172	0.015	0.569	1.131	13.6	0.249
C. C.	0.053	0.208	0.115	0.199	0.019	0.598	1.228	13.4	0.274
N. C.	0.058	0.428	0.310	0.387	0.064	0.615	1.865	58.2	0.511
				Subacute Lymphocytic Leukemia					
R. F.	0.082	0.278	0.174	0.146	0.016	0.519	1.195	9.4	0.432
				Acute Lymphocytic Leukemia					
T. M.	0.087*	0.347*	0.265*	0.345*	0.028*	0.742*	1.804*	48.6	0.565
T. M.	0.118	0.350	0.270	0.357	0.029	0.835	1.947	68.8	0.530
T. M.	0.059	0.297	0.216	0.257	0.022	0.648	1.470	56.2	0.388
T. M.	0 070	0 353	0.253	0.303	0.026	0.757	1.743	66.2	0.457

*These determinations were made prior to treatment with P^{32}.

significant differences were observed, the lowest probability being that of the inorganic-P in chronic lymphocytic leukemias (P—0.33). Sex and age were also found to exert no significant effect on the phosphorus fractions.

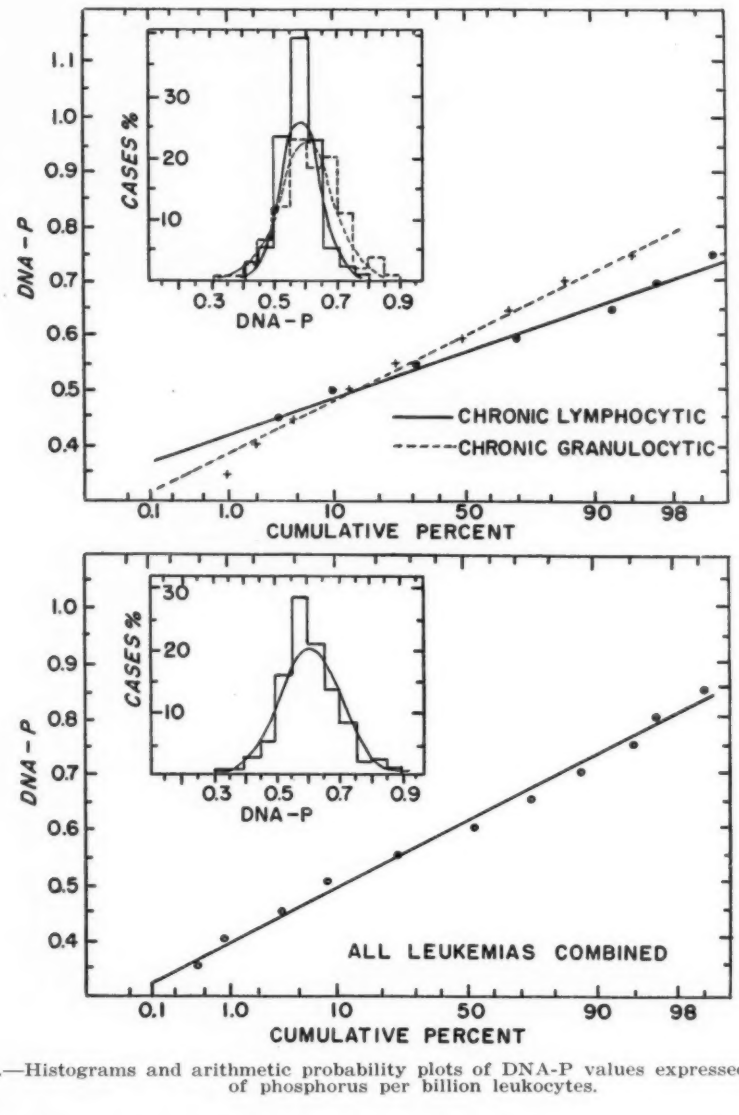

Fig. 1.—Histograms and arithmetic probability plots of DNA-P values expressed in milligrams of phosphorus per billion leukocytes.

Analysis of variance disclosed that in chronic granulocytic leukemias, the RNA-P, acid soluble organic-P, and phospholipid-P fractions are the only ones which differ significantly at the 1 per cent level among patients; while in the chronic lymphocytic leukemias, all fractions except the DNA-P are significantly different among patients. The small number of observations in the other types of leukemia precludes such analysis. The combined data or the weighted means from all patients were found to be normally distributed. That this may be true

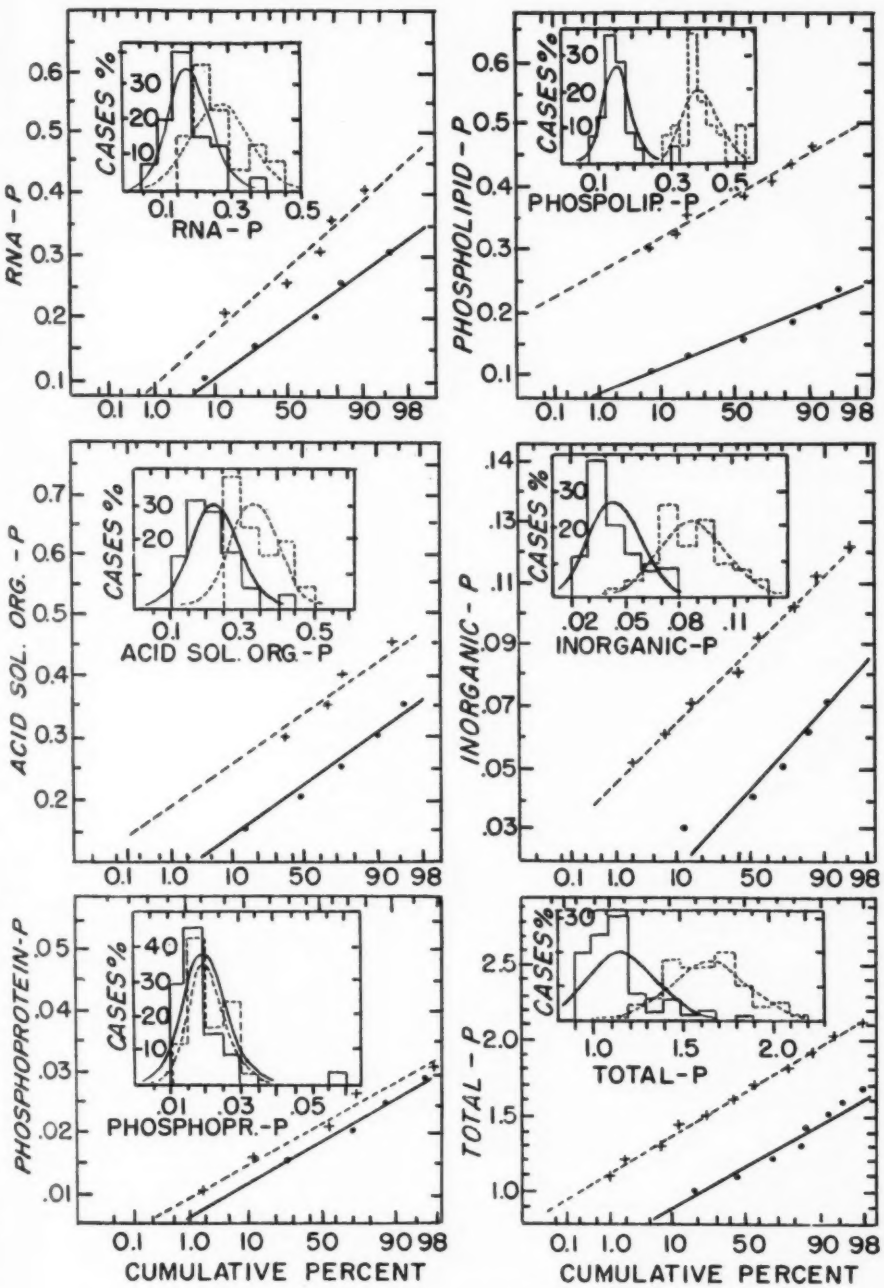

Fig. 2.—Histograms and arithmetic probability plots of the phosphorus fractions expressed in milligrams of phosphorus per billion leukocytes.

is also evident in Figs. 1 and 2 from the straight lines obtained on arithmetic probability paper for each fraction, and from the reasonable good fit of the Gaussian distribution to the corresponding histograms. A case of eosinophilic leukemia (Patient W. S.) has been included in the group of chronic granulocytic leukemias, since the values obtained in this case were found by the Dixon criterion[18] not different at the 5 per cent level of significance from the chronic granulocytic group. Similarly, the values obtained from a case of subacute lymphocytic leukemia (Patient R. F.) were assigned to the population of chronic lymphocytic leukemias. Arithmetic means and standard deviations for fractions not significantly different among patients, as well as weighted means and standard deviations for the others, were calculated for the chronic granulocytic and chronic lymphocytic leukemias as explained under methods. Arithmetic means and standard deviations for the acute lymphocytic and monocytic leukemias were also calculated from the limited number of determinations. The results are tabulated in Table V. From this table it can be seen that the DNA-P is very similar in all types of leukemia (analysis of variance failed to show any significant difference at the 1 per cent level of significance). All the DNA-P determinations were therefore pooled, giving a mean DNA-P value of 0.607 mg. per billion leukocytes (Table V). The rest of the fractions were

TABLE V. PHOSPHORUS MEAN VALUES IN HUMAN LEUKEMIC LEUKOCYTES

PHOSPHORUS FRACTION	CHRONIC GRANULOCYTIC LEUKEMIA	CHRONIC LYMPHOCYTIC LEUKEMIA	ACUTE LYMPHOCYTIC LEUKEMIA	ACUTE AND SUBACUTE MONOCYTIC LEUKEMIA	ALL LEUKEMIAS COMBINED
	Milligrams of Phosphorus Per 10^9 Leukocytes				
Total-P	1.649 (0.217)* $n = 95$	1.156† (0.204†) $n' = 29$	1.424 (0.274) $n = 12$	1.920 (0.340) $n = 16$	
DNA-P	0.617 (0.087) $n = 104$	0.577 (0.061) $n = 102$	0.638 (0.098) $n = 12$	0.663 (0.080) $n = 17$	0.607 (0.095) $n = 235$
RNA-P	0.269† (0.119†) $n' = 15$	0.185† (0.055†) $n' = 15$	0.316 (0.045) $n = 4$	0.450 (0.092) $n = 3$	
Phospholipid-P	0.377† (0.050†) $n' = 15$	0.152† (0.044†) $n' = 15$	0.251 (0.021) $n = 4$	0.311 (0.050) $n = 3$	
Acid sol. org.-P	0.335† (0.064†) $n' = 15$	0.223† (0.117†) $n' = 15$	0.338 (0.027) $n = 4$	0.371 (0.070) $n = 3$	
Phosphoprotein-P	0.021 (0.004) $n = 42$	0.019† (0.009†) $n' = 15$	0.026 (0.003) $n = 4$	0.036 (0.004) $n = 3$	
Inorganic-P	0.087 (0.019) $n = 42$	0.044† (0.014†) $n' = 15$	0.084 (0.026) $n = 4$	0.111 (0.051) $n = 3$	

n is the number of observations (determinations) on which the mean is based.

n' is the number of observations (patients) on which the mean is based.

*Figures in parentheses represent the standard deviation of a single determination on a random patient.

†These statistics have been weighted as explained in the text.

found to be much higher in the chronic granulocytic than in the chronic lymphocytic leukemias ($P < 0.0001$), with the exception of the phosphoprotein-P which is not significantly different. A certain degree of overlapping of the values obtained in the two types of leukemias is evident in the histograms in Fig. 2, ranging from complete overlapping of the phosphoprotein-P to a minimum of the phospholipid-P. Although the small number of determinations on acute lymphocytic and monocytic leukemias precludes definite conclusions, it appears that leukocytes from such leukemias are very similar in their phosphorus composition to those from chronic granulocytic leukemias.

Amount of Phosphorus Compounds Per Milliliter of Human Leukemic Leukocytes.—The values of the phosphorus fractions, when calculated as milligrams of P per milliliter of leukocytes, are found to be significantly different among patients for every fraction, including the DNA-P. The weighted means and standard deviations, calculated by the formulas previously given, are shown in Table VI. A comparison of the mean values obtained in chronic granulocytic leukemias with those of chronic lymphocytic leukemias by the "t" test revealed that the former contain significantly more phospholipid-P, but significantly less total-P, DNA-P, RNA-P, acid soluble organic-P, and phosphoprotein-P ($P < 0.0001$). The inorganic-P is not significantly different at the 1

TABLE VI. PHOSPHORUS MEAN VALUES IN HUMAN LEUKEMIC LEUKOCYTES

PHOSPHORUS FRACTION	CHRONIC GRANULOCYTIC LEUKEMIA	CHRONIC LYMPHOCYTIC LEUKEMIA	ACUTE LYMPHOCYTIC LEUKEMIA	ACUTE AND SUBACUTE MONOCYTIC LEUKEMIA
	Milligram of Phosphorus Per Milliliter of Leukocytes			
Total-P	3.254* (0.351*) $n = 26$	4.728* (0.594*) $n = 29$	3.889* (0.370*) $n = 3$	3.718* (0.259*) $n = 6$
DNA-P	1.205* (0.180*) $n = 26$	2.439* (0.599*) $n = 29$	1.758* (0.227*) $n = 3$	1.380* (0.148*) $n = 6$
RNA-P	0.482* (0.119*) $n = 15$	0.648* (0.106*) $n = 15$	0.653 (0.029)† $n' = 4$	0.790* (0.101*) $n = 2$
Phospholipid-P	0.681* (0.092*) $n = 15$	0.546* (0.059*) $n = 15$	0.523 (0.042)† $n' = 4$	0.599* (0.046*) $n = 2$
Acid sol. org.-P	0.596* (0.076*) $n = 15$	0.804* (0.117*) $n = 15$	0.706 (0.079)† $n' = 4$	0.712* (0.072*) $n = 2$
Phosphoprotein-P	0.038* (0.008*) $n = 15$	0.064* (0.016*) $n = 15$	0.054 (0.004)† $n' = 4$	0.071* (0.001*) $n = 2$
Inorganic-P	0.159* (0.032*) $n = 15$	0.154* (0.029*) $n = 15$	0.170 (0.035)† $n' = 4$	0.167* (0.104*) $n = 2$

n is the number of observations (patients) on which the mean is based.

n' is the number of observations (determinations) on which the mean is based.

*These statistics have been weighted as explained in the text. Figures in parentheses represent the standard deviation of the mean of several determinations on a random patient.

†The numbers in parentheses represent the standard deviation of a single determination on a patient.

TABLE VII. RELATION OF PHOSPHORUS CONTENT TO MATURITY OF HUMAN LEUKEMIC LEUKOCYTES

CHRONIC GRANULOCYTIC LEUKEMIA						
PHOSPHORUS FRACTION	MULTIPLE REGRESSION COEFFICIENT	PARTIAL STANDARD REGRESSION COEFFICIENTS		REGRESSION EQUATION*	MEAN PHOSPHORUS CONTENT (MG. P/10^9 CELLS)	
		$b'_{Y1.2}$	$b'_{Y2.1}$		CELLS WITH NUCLEOLI	CELLS WITHOUT NUCLEOLI
RNA-P	0.88 $P < 0.01$†	0.70 ± 0.17‡ $P < 0.0001$	0.40 ± 0.19 $P < 0.0001$	$\hat{Y} = 0.066 + 0.006X_1 + 0.233X_2$	0.760 ± 0.134	0.200 ± 0.015
Acid sol. org.-P	0.84 $P < 0.01$	0.30 ± 0.18 $P < 0.001$	0.78 ± 0.18 $P < 0.0001$	$\hat{Y} = 0.031 + 0.003X_1 + 0.491X_2$	0.582 ± 0.154	0.304 ± 0.017
		$b'_{Y3.2}$	$b'_{Y2.3}$		Blast to band	Segmented
Phospholipid-P	0.70 $P < 0.01$	0.39 ±0.24 $P < 0.001$	0.39 ± 0.24 $P < 0.001$	$\hat{Y} = 0.225 + 0.002X_3 + 0.212X_2$	0.343 ± 0.025	0.492 ± 0.066

LYMPHOCYTIC LEUKEMIA				
PHOSPHORUS FRACTION	STANDARD REGRESSION COEFFICIENT (b'_{YX})	REGRESSION EQUATION*	MEAN PHOSPHORUS CONTENT (MG. P/10^9 CELLS)	
			LYMPHOBLASTS AND PROLYMPHOCYTES	MATURE LYMPHOCYTES
RNA-P	0.69 ± 0.01 $P < 0.0001$	$\hat{Y} = 0.125 + 0.004X$	0.537 ± 0.005	0.125 ± 0.001
Acid sol. org.-P	0.65 ± 0.01 $P < 0.0001$	$\hat{Y} = 0.165 + 0.004X$	0.541 ± 0.004	0.165 ± 0.001
Phospholipid-P	0.74 ± 0.005 $P < 0.0001$	$\hat{Y} = 0.105 + 0.003X$	0.426 ± 0.003	0.105 ± 0.001
Inorganic-P	0.54 ± 0.002 $P < 0.0001$	$\hat{Y} = 0.036 + 0.0006X$	0.100 ± 0.001	0.036 ± 0.0003
Phosphoprotein-P	0.54 ± 0.002 $P < 0.0001$	$\hat{Y} = 0.014 + 0.0003X$	0.042 ± 0.0006	0.014 ± 0.0002

*$\hat{Y}$ is the predicted amount of the phosphorus fraction; X_1 is the percentage of cells with nucleoli; X_2 is the size of the cells in milliliters per 10^9 cells; X_3 is the percentage of segmented cells; X is the percentage of lymphoblasts plus prolymphocytes.
†*P* values indicate the chance probability of a regression coefficient of this magnitude arising from a system having zero regression.
‡The ± figures give the 95 per cent confidence limits for the mean.

per cent level of significance. Again no definite conclusions can be reached about any possible differences that may exist among the other types of leukemia because of the insufficient number of determinations.

Correlation of the Phosphorus Fractions With Maturity and Size of the Leukocytes.—

a. *Chronic Granulocytic Leukemias.*—It would be of interest to know if the differences which were observed in the RNA-P, acid soluble organic-P, and phospholipid-P among patients with chronic granulocytic leukemia are correlated with the percentages of different maturation stages and with the size of the leukocytes, both of which were also found to differ significantly among patients and to be independent of each other. Multiple regression analysis was, therefore, done. The size of the cells and the percentages of cells with nucleoli (myeloblasts, progranulocytes, and early myelocytes), late myelocytes (myelocytes devoid of nucleoli), metamyelocytes, band cells, and segmented cells were considered six independent predicting variables. The relatively small percentage (approximately 4 per cent) of cells other than those of the granulocytic series and the disintegrated cells were not included among the independent predicting variables, since the first did not vary significantly among patients ($P \sim 0.60$), while the latter is not independent, being determined from the percentages of the other cells. This analysis resulted in multiple regression coefficients significantly different from zero at the 1 per cent level of significance for all three fractions. However, from the partial standard regression coefficients, only those for the cell size and for the nucleoli-possessing cells in the case of the RNA-P and acid-soluble organic-P, and those for the cell size and for the segmented cells in the case of the phospholipid-P were found to be significantly different from zero at this level of significance. The nonpredicting variables were, therefore, eliminated and the analysis repeated.

The regression coefficients, their 95 per cent confidence limits, and probabilities (P) are shown in the first three columns of Table VII. Regression equations which predict the amount of each phosphorus fraction from the cell size and the per cent of cells with nucleoli, or the per cent of segmented leukocytes, were derived and are shown in Table VII. Assuming average cell size, it was possible to calculate the predicted mean values of phosphorus fractions in leukocytes at different stages of differentiation. These are shown in Table VII. It is interesting to note that the nucleoli-bearing immature granulocytes were estimated to contain about four times as much RNA-P and twice as much acid soluble organic-P as the nucleoli-devoid mature granulocytes. Phospholipid-P on the other hand is seen to be more abundant in the segmented mature granulocytes.

b. *Lymphocytic Leukemias.*—In the chronic lymphocytic leukemias, every phosphorus fraction except the DNA-P was found to differ significantly among patients. As in the case of the chronic granulocytic leukemias, significant differences among patients were found to exist at the 1 per cent level in size and percentages of cells at different stages of differentiation. In this case, however, a definite correlation of size with stage of differentiation was found, with a re-

gression coefficient equal to 0.72 ($P < 0.0001$), which led to the regression equation $\hat{Y} = 0.232 + 0.004X$, where $\hat{Y}$ is the predicted cell size and X the percentage of lymphoblasts plus prolymphocytes. Cell size, therefore, was not taken as an independent variable. The small percentage (around 1.5 per cent) of cells other than lymphocytic series and percentage of disintegrated cells were again not considered as independent predicting variables for similar reasons to those given for the chronic granulocytic leukemias. From the remaining two predicting variables, i.e., the percentage of the lymphoblasts plus prolymphocytes, and the percentage of the mature lymphocytes, only the first gave significant regression coefficients at the 1 per cent level of significance. Following elimination of the second variable, the regression coefficients, regression equations, and predicted mean values of the phosphorus fractions of lymphoblasts and prolymphocytes versus those of the mature lymphocytes were determined and are shown in Table VII. It was thus revealed that in the chronic lymphocytic leukemias, in contrast to the chronic granulocytic leukemias, every phosphorus fraction except that of the DNA-P decreases with cell maturity.

DISCUSSION

Various phosphorus fractions of the human leukocytes have been studied in the past by various investigators, but only the recent report by Lawrence[19] gives values for every phosphorus fraction of the human leukemic leukocytes. Unfortunately, these values are given as percentages of normal which makes them difficult to interpret and impossible to compare with our values. The fractions which have been most extensively studied are those of the nucleic acids, the Schmidt and Thannhauser method being the most commonly used for this purpose. The constancy of the DNA in diploid nonpolytene nuclei of each species,[20, 21] which supports the concept of its close relationship to the genes, has stimulated the study of the DNA content of human blood leukocytes.[22-29] It was thus found that the amount of DNA per cell is similar in both normal and leukemic leukocytes. The reported DNA-P values, derived from a relatively small number of determinations, range from slightly higher (but not significantly so [$P > 0.64$]) to slightly lower than the value of 0.607 mg. of DNA-P per 10^9 leukocytes which was determined in this laboratory from 235 analyses. Leukocytes from normal and leukemic human marrow have also been studied,[24, 29-37] and these reports agree as to the amount of DNA per cell, but the values are consistently higher than for leukocytes from blood.[26] The higher DNA values encountered in leukocytes from marrow have been attributed to the presence of cells building up DNA just prior to mitosis,[38] to polyploidy, or polyteny.[37] The decrease of DNA content per marrow leukocyte following x-ray treatment[36] may reflect a decrease in the number of cells preparing for mitosis.

There are reasons to believe that the DNA content of the blood leukocyte may be the closest approximation to that of the diploid nonpolytene human cell that has thus far been derived by direct chemical determination. Supporting evidence for this belief is provided by the fact that the DNA

content of the haploid nucleus of normal human spermatozoa, which is uniformly constant,[39] was found to be closer to half the DNA content of the blood leukocytes[32, 37] than to that of the marrow leukocytes or other human tissues.[32, 40] In addition Marinone[41] and Petrakis,[42] utilizing the microspectrophotometric technique developed by Caspersson,[43] observed that normal blood lymphocytes have a highly uniform content of DNA, which is twice the amount of the human spermatozoa, in contrast to lymphocytes from lymph nodes or bone marrow, many of which possess four times as much (tetraploid) or intermediate amounts (aneuploid) of DNA. Very few of the chronic lymphocytic leukemias, an appreciable number of the chronic granulocytics, and most of the acute leukemias were found by these investigators to have leukocytes circulating in the blood possessing more than twice the DNA of the human spermatozoa. On the basis of these observations, one would expect that the mean DNA content per leukocyte would be lowest and closest to that of a diploid nonpolytene human cell in the chronic lymphocytic leukemias, and highest in the acute leukemias. Although an inspection of the data tabulated in Table V indicates that this may be true, the data in the acute leukemias are not sufficient to reach a definite conclusion.

The RNA content of the cells has been the subject of thorough investigation since Caspersson[44] pointed out its relationship to protein synthesis, a thesis which is now strongly supported by the work of Gale and Folkes.[45] The RNA content of human blood and marrow leukocytes frequently has been chemically determined.[23, 25-37] Davidson observed significantly higher RNA values in leukemic blood leukocytes than in normal leukocytes and a trend toward higher values in the acute versus the chronic leukemias, which is suggestive of a decrease of the RNA content with cell maturity. He also observed higher RNA values in marrow than in normal blood leukocytes but no difference between normal and leukemic marrow leukocytes. It is difficult to compare the RNA values given by these investigators with ours, because of the existing differences among patients and among leukemias (vide supra).

The relationship of the RNA content of cells of the hemopoietic system to their growth rate and protein synthesis has been studied by microspectrophotometric techniques by Thorell,[46] who observed a decrease in cytoplasmic nucleotides in the mature cells and concluded that the RNA content decreases with cell maturity. This conclusion is confirmed by the results obtained in this department from direct chemical determinations.

The phospholipid fraction of human leukocytes has been determined by Byrom and Kay[47] and Boyd,[48-56] who reported values which when converted to phospholipid phosphorus are similar to ours. Boyd[52, 54, 55] observed, as we did, the statistically significant lower phospholipid content of the lymphocytes. The increase of phospholipid-P with increased cell maturity which we observed (vide supra) in the granulocytic leukemias may be related to the accumulation of cytoplasmic granules, which are known to contain phospho-

lipids,[57] while the corresponding decrease with cell maturity in the lymphocytic leukemias may be due, at least in part, to the smaller size of the mature lymphocytes.

The literature on acid-soluble phosphorus compounds of human leukocytes is very meager. Abels, and associates,[58] who studied the effect of x-ray and P^{32} treatment on these compounds, observed a variable rise, followed by a gradual drop, after initiation of treatment when these compounds were expressed as milligrams of P per milliliter of leukocytes. An increase in ATP per million leukocytes following x-ray therapy of a patient was observed by Schwartz,[59] without any appreciable change in quantity of total acid-soluble phosphorus compounds. Changes similar to those observed by Abels were apparent in some of our patients when the data were expressed per milliliter of leukocytes, but they were not consistent. Such changes were invariably accompanied by a parallel change in every phosphorus fraction and an opposite change in the size of the cells; the latter may explain the observed changes of the former.

As in the case of the acid-soluble phosphorus, the data reported in the literature on total leukocyte phosphorus are very scanty. A small number of determinations were performed by Larizza,[60] Schwartz,[59] and Waterhouse and associates.[61] The reported values expressed in milligrams of P per milliliter of leukocytes fall in the same range as ours.

We were unable to find any data in the literature on the phosphoprotein fraction of human leukocytes.

The phosphorus fractions which have been determined by chemical analyses represent the mean values of a population of cells at all stages of differentiation. Differences in the phosphorus fractions among leukocytes at different stages present in various samples would thus result in different mean values (vide supra), the frequency distribution of which will reflect the distribution of the differential counts of patients with leukemia. The weighted mean of such a distribution should, therefore, be used with caution, since not every patient could be expected to yield this mean. This applies to the RNA-P, acid-soluble organic-P, and phospholipid-P in the chronic granulocytics and all fractions except the DNA-P in the lymphocytic leukemias, i.e., the fractions found to be correlated with the maturity of the cells.

As previously mentioned, the decrease in the phosphorus fractions in the lymphocytic leukemias could be explained in part by the concomitant decrease in the cell size. In the chronic granulocytic leukemias no correlation of the size with maturity of the cells was observed in this group of patients as a whole. This, however, does not exclude the possibility of the existence of such a correlation for each individual patient, similar to that observed by Thorell[46] in normal leukocytes of the granulocytic series. Size of the cells, nevertheless, proved to be one of the determining factors for the RNA-P, acid soluble organic-P, and phospholipid-P content of the leukocytes. More important, however, from the standpoint of cell growth and differentiation is the observed correlation of these phosphorus compounds with the maturity of

the cell. It is quite intriguing that the immature, nucleoli-possessing cells, which are considered the only ones capable of division, were found to contain four times as much RNA (essential in protein synthesis[45]) and twice as much acid-soluble organic phosphorus compounds (key substances in general and energy metabolism and in growth of the cell[62]) as the mature cells that are devoid of nucleoli and have lost their ability to divide. The phospholipids, on the other hand, which are thought to be an integral part of many enzymes that participate in the oxidative metabolic processes,[63] were found to be much higher in the segmented (the most mature) granulocytes. Although the statistical demonstration of these correlations does not constitute a proof that they have a real basis, the agreement with those anticipated from the growth and metabolic characteristics of the cells as well as with the size of the cells, induces us to believe that they may not be a mere mathematical deception.

SUMMARY

The phosphorus compounds of human leukemic blood leukocytes, obtained from 62 patients with various types of leukemia, were determined in replicate by the Schmidt and Thannhauser procedure. Results expressed in milligrams of P per billion leukocytes showed a constancy in the mean DNA-P content among patients and among leukemias. This value was 0.607 mg., with a standard deviation of 0.095. The mean values and standard deviations (in parentheses) of the other phosphorus fractions in chronic granulocytic leukemia were: RNA-P, 0.269 (0.119); acid-soluble organic-P, 0.335 (0.064); phospholipid-P, 0.377 (0.050); phosphoprotein-P, 0.021 (0.004); inorganic-P, 0.087 (0.019); and total-P, 1.649 (0.217). In chronic lymphocytic leukemia these fractions were significantly lower (except for the phosphoprotein-P) and are: 0.185 (0.055); 0.223 (0.117); 0.152 (0.044); 0.019 (0.009); 0.044 (0.014); and 1.156 (0.204), respectively. Acute lymphocytic and monocytic leukemias showed values similar to those of chronic granulocytic leukemia. The RNA-P, acid-soluble organic-P, and the phospholipid-P were found to be significantly different among patients with chronic granulocytic leukemia. All phosphorus fractions with the exception of the DNA-P differ significantly among patients with chronic lymphocytic leukemia. These were found by a multiple regression analysis to be correlated with the size and maturity of the cells, increasing with size and decreasing with maturity, with the exception of the phospholipid-P in chronic granulocytic leukemias, which increases with cell maturity. It was possible to estimate the content of these fractions in leukocytes at different stages of maturation, which indicates a fourfold and a twofold decrease in the RNA-P and in the acid-soluble organic-P, respectively, accompanied by an increase in the phospholipid-P of the mature granulocytes, while mature lymphocytes show an approximate fourfold decrease in every phosphorus fraction (except DNA-P). The implications of these correlations for growth and differentiation of the leukocytes and the limitations of the methods are discussed.

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THE EFFECT OF INTRAVENOUS FAT EMULSIONS ON TOTAL BILIRUBIN OUTPUT AS A MEASURE OF HEMOLYSIS IN HUMAN SUBJECTS

JOHN F. MUELLER, MAJOR, MC, USAR, MORTON I. GROSSMAN, M.D., PH.D.,* AND HUGO C. MOELLER, M.D., PH.D.**
DENVER, COLO.

INVESTIGATIONS into the preparation of a suitable emulsion of fat for intravenous alimentation have been carried out for many years. The early problems of stability and satisfactory particle size have, in a large measure, been overcome; yet such an emulsion is not available for general use today The reason for this is the high incidence of adverse reactions in human subjects. The incidence of such reactions varies from place to place probably as a result of a combination of factors. Thus, composition of emulsion, type of patient, criteria of reaction, and many other variables probably account for the discrepancy of reaction rates from various laboratories interested in this problem.

There is agreement, however, that fever is the most common reaction encountered when administering intravenous fat emulsions. In our series of 110 patients receiving one or more infusions, 34 per cent developed a temperature over 100.6° F. orally or 101.6° F. rectally; approximately half of these patients also had an associated chill. Studies in this laboratory[1] have shown no correlation in animals between fever and the following variables: free fatty acid content of the emulsion; the levels of peroxide and carbonyl radicles; particle size; rapid clearing with heparin; aging or autoclaving of the emulsion. It has been suggested that endogenous heat production as a result of the metabolism of the large amount of fat might be responsible for the fever.[2] However, the inconsistencies of response from patient to patient make this hypothesis untenable. Phosphatide impurities also have been blamed.[3] Hemolysis as a cause of the fever has been the most prominent theory expressed.

The in vitro hemolytic activity of free fatty acids has long been recognized. Freeman and Johnson,[4] in 1940, showed increased red cell fragility and hemo-

From the U.S. Army Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colo.

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*Present address: Veterans Administration Center, Wilshire and Sawtelle Boulevards, Los Angeles 25, Calif.

**Present address: Veterans Administration Research Hospital, 333 East Huron Street, Chicago, Ill.

globinemia as a result of alimentary lipemia and ascribed the hemolytic activity of lipemic chyle to its free fatty acid and soap content. Intravascular hemolysis and severe reactions were reported by Shafiroff, Mulholland, and Baron[5] to occur after the infusion of relatively soluble short-chained glycerides derived from coconut oil. More recently, Creditor[6] has shown remarkable increases in mechanical fragility of the red cell, increases in plasma hemoglobin, and inconsistent small increases in fecal urobilinogen in two patients receiving fat intravenously. He suggests that in some patients hemolysis may be the cause of fever. Previous studies by other workers failed to show these alterations.[7-9]

Since tremendous increases in mechanical fragility of the red blood cell do occur in some subjects, the measurement of plasma hemoglobin may merely reflect the trauma incurred in the drawing of the blood sample. Thus a "pseudohemoglobinemia" may be confused with true intravascular hemolysis. Therefore, measurements of hemoglobin degradation products seemed to be a more direct approach to the problem. The vagaries of the fecal urobilinogen test are well recognized. Thus, direct measurements of bilirubin in bile were first performed in animals.

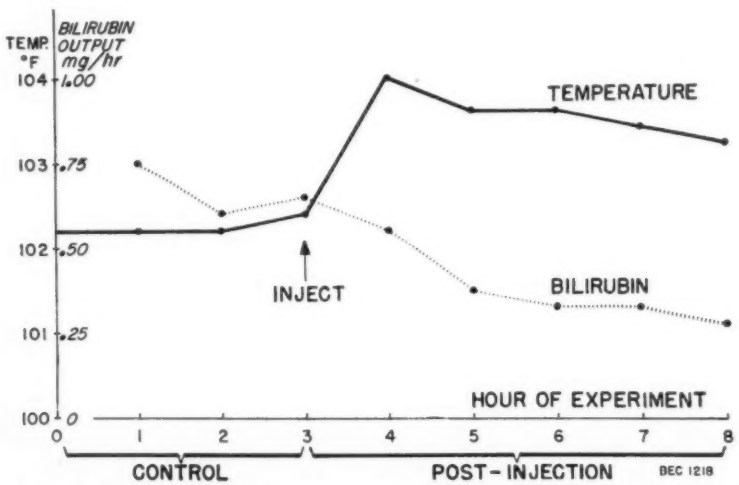

Fig. 1.—Effect of intravenous injection of 10 per cent fat emulsion on temperature response and bilirubin output of rabbits.

Becker and Grossman[10] from this laboratory reported on the effect of intravenous fat on the bilirubin output of the rabbit in which a bile fistula had been surgically induced (Fig. 1). In these animals which received a 10 per cent fat emulsion in the dose of 10 ml. per kilogram there was no elevation of the bilirubin output following fat, although the usual febrile response occurred. More recent work from this laboratory, utilizing rats in which the bile is obtained by surgical cannulation of the bile duct, revealed that increases of bilirubin do occur with excessively high doses of intravenous fat and that this is directly proportional to the dose level (Table I). From the latter

studies it would appear that intravenous fat is capable of producing in vivo hemolysis in the rat in high dosage levels, much higher than those given to human beings.

Since species differences are well recognized, it seemed imperative to extend these observations to human beings. The results of these studies are the basis of this report.

TABLE I. AVERAGE MICROGRAMS OF BILIRUBIN EXCRETED PER RAT PER HOUR, BEFORE AND FOLLOWING INTRAVENOUS FAT EMULSION

Dose (ml./Kg.)	10	20	40
Number of rats	8	9	9
Before	21.3	44.7	50.9
After	25.3	60.1	125.6

MATERIALS AND METHODS

Eleven patients in the Colorado General Hospital and the Denver Veterans Administration Hospital were studied. Ten of these patients were recuperating from cholecystectomy and common duct exploration and had bile draining freely from an indwelling T-tube. The other patient had a spontaneous biliary fistula as a result of surgery performed in another hospital.

The bile was collected for 48 hours before and 48 hours after an infusion of 600 ml. of Lipomul, a 15 per cent cottonseed oil emulsion.* The collections were divided into four 3-hour periods during the day and one overnight 12-hour period. Each specimen was analyzed for bilirubin according to the method of Malloy and Evelyn,[11] and the total output in milligrams was calculated for each collection period. These individual values were then added and expressed as total output of bilirubin in milligrams per 24-hour period.

Fig. 2.—The total milligrams of bilirubin in each collection period in a representative subject for the four-day study period.

The entire output of bile was not collected from the T-tubes, since the patients were passing brown stools. However, the figures indicate that the quantity of bilirubin from individual patients varied in proportion to the volume of bile excreted (Table II and Fig. 2). This gross correlation between volume of bile and content of bilirubin suggests that the concentration of the latter remains fairly constant in relation to the volume of bile excreted. If this is so, then any deviation in the output of bilirubin as a result of the experimental procedure would be reflected in the aliquot obtained via the indwelling T-tube, regardless of what per cent of the total bile output is recovered. Furthermore, from figures on total output of bile in the literature, it would appear that a large percentage of the bile was collected.

*We wish to express our gratitude to Drs. E. A. Hawk and R. Heinle of The Upjohn Company for their generosity in supplying us with Lipomul.

TABLE II

	DAY	1						2						3						4					
	SPECIMEN NO.	1	2	3	4	5	TOTAL 24 HOURS	1	2	3	4	5	TOTAL 24 HOURS	1	2	3	4	5	TOTAL 24 HOURS	1	2	3	4	5	TOTAL 24 HOURS
C. B.	ml. bile	48	44	53	45	131	315	51	65	66	65	266	520	53	31	41	59	199	367	62	76	73	62	190	463
	mg. bilirubin	21.7	22.1	26.7	26.2	113	209.7	37.5	33.7	26.4	26.5	117.6	247.8	22.7	16.3	17.7	25.9	81.7	157.5	21.5	24.6	24.6	22	98.4	191.5
D. H.	ml. bile	52	47	85	45	145	374	49	74	42	64	128	359	51	65	94	21	154	427	64	86	81	60	170	461
	mg. bilirubin	40.3	34.5	45.3	25.6	92.4	238.5	38.9	36.8	20.2	35.5	87.2	218.4	37.4	35.3	27.3	8.8	78.2	204.9	31.7	41	32.2	23.8	82.8	212
J. K.	ml. bile	96	73	95	56	260	580	80	93	68	70	271	582	68	52	74	56	263	513	59	73	74	52	250	508
	mg. bilirubin	54.7	40.2	48.4	40.3	161	344.6	53.6	42.8	51.7	44.1	176	368.2	40.1	26	34.8	24.6	179	304.5	32.4	32.8	34.8	31.7	112.5	259.1
T. P.	ml. bile	10	62	70	62	142	346	101	72	92	42	340	647	56	49	70	55	239	469	62	70	64	42	260	498
	mg. bilirubin	4.9	22.9	25.9	22.9	96.5	173.1	56.6	49.7	46.9	26.4	180	359.6	38.1	27.9	39.9	29.2	136	271.1	41.5	34.3	35.2	25.6	140	276.6
M. D.	ml. bile	69	60	68	42	356	575	72	64	74	58	226	494	40	54	52	32	163	341	70	68	82	60	242	522
	mg. bilirubin	48.3	40.2	43.5	26	195.8	368	33.8	32.6	39.2	28.4	111	245	17.2	23.8	28.6	14.4	63.6	147.6	28	22.4	32	22.2	104	208.6
J. M.	ml. bile	8	12		12	182	238	45	48	64	30	181	338	56	31	30	32	269	418	90	74	64	54	222	504
	mg. bilirubin	4.2	5.5		3.2	98.3	134.7	22.5	22.1	33.9	14.4	99.5	192.4	30.2	14.3	18.3	16.3	143	222.1	47.7	34	30.7	24.8	113	250.2
S. M.	ml. bile	30	42	34	34	137	277	44	50	54	50	225	423	50	45	55	60	200	410	54	56	54	65	245	474
	mg. bilirubin	63	90.5	77.5	56	260	547	52	97	54.5	43.5	205	452	53	27	35	32	118	265	32	30	29	35	137	263
J. N.	ml. bile	50	52	50	50	189	391	55	55	54	54	190	408	49	40	50	45	160	344	50	55	55	57	178	395
	mg. bilirubin	84.5	78.5	78.5	79.5	293	614	96	79.2	80.5	77.8	294	627.5	74.5	46.8	54.5	40.5	251	467.3	83.5	77	73.7	78.6	238	550.8
E. G.	ml. bile	60	72	30	85	265	512	40	58	56	32	225	411	75	70	65	70	195	475	50	40	40	55	225	410
	mg. bilirubin	19.2	13.7	6.6	17.0	66.2	122.7	9.6	12.8	10.6	5.8	60.7	99.5	25.5	16.8	14.3	15.4	62.7	134.4	15	7.6	7.6	10.45	60.8	101.5
B. S.	ml. bile		52	60	51	226	439	66	60	50	55	233	464	54	50	30	44	227	405	64	50	52	46	260	472
	mg. bilirubin		65	74.4	63.8	278	544.4	74	68.4	59	64.4	282	547.8	62.1	43.5	26.4	38.3	245	392.8	67.8	38.5	41.6	36.3	229	413.2
Z. H.	ml. bile	54	68	50	52	172	396	27	50	48	50	205	403	60	47	44	50	195	396	60	54	50	52	230	446
	mg. bilirubin	76	114	72.5	75	266	603.5	36.7	59.5	57.5	74.5	264	512.2	57	40.4	39.2	47.5	195	379.1	60.6	49.1	44.5	43.7	207	404.9

Absolute values for volume of bile and milligrams of bilirubin in individual specimens collected from eleven patients with T-tubes in the common duct.

Each received 600 ml. I.V. fat emulsion on the morning of the third day.

Specimen No. 1, 7:30 A.M.–10:30 A.M.; No. 2, 10:30 A.M.–1:30 P.M.; No. 3, 1:30 P.M.–4:30 P.M.; No. 4, 4:30 P.M.–7:30 P.M.; No. 5, 7:30 P.M.–7:30 A.M.

RESULTS

The actual values obtained are reproduced in Table II for each collection period.

The total 24-hour output of bilirubin is depicted for each individual in Figs. 3, 4, and 5. In only 2 of the 11 patients was there any increase in bilirubin after the intravenous emulsion. These two patients were the only ones of the group who had gross and microscopic evidence of liver disease at the

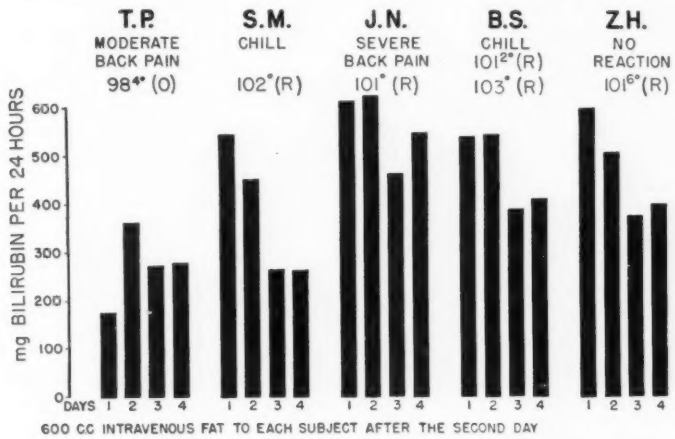

Fig. 3.—Total 24-hour bilirubin values expressed in milligrams for two days before and two days after 600 ml. of intravenous fat emulsion. The recorded temperature indicates the highest elevation reached during and for 12 hours after the infusion.

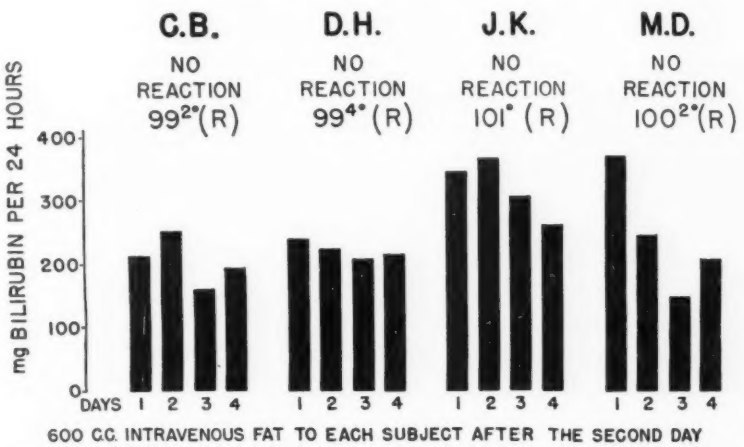

Fig. 4.—Total 24-hour bilirubin values expressed in milligrams for two days before and two days after 600 ml. of intravenous fat emulsion. The recorded temperature indicates the highest elevation reached during and for 12 hours after the infusion.

time of operation. The pathologic report on the liver biopsy of E. G. showed "cirrhosis, type undetermined" and the biopsy of J. M. showed "cholangitis, subacute and chronic." In addition, the increases of bilirubin postinfusion were of such magnitude that they were not statistically significant. J. M. was also the only one of the group who was clinically, as well as chemically,

icteric at the time of the study and the progressively rising bilirubin output was taken as evidence of clearing jaundice in a patient with a diseased liver recovering from the effects of common duct obstruction.

In 9 of the 11 patients there was no significant change. The slight decreases were not of statistical significance and probably represent responses of bilirubin clearing from a previously partially obstructed biliary system by a normally functioning liver.

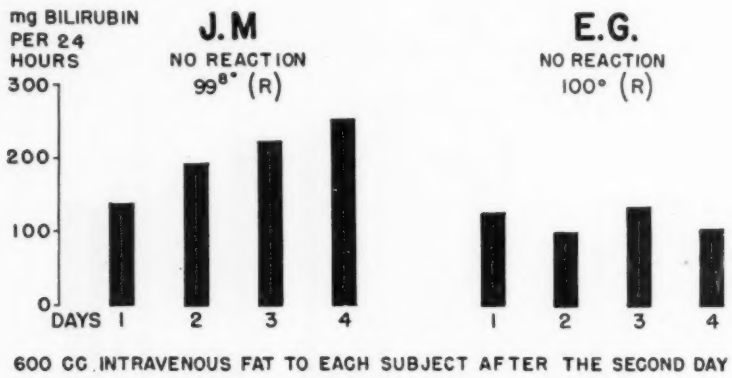

FIG. 5.—Daily bilirubin output in two patients with liver disease diagnosed by biopsy at operation.

DISCUSSION

Although studies in animals have shown that the measurement of bilirubin in bile collected from surgically induced biliary fistulas is an accurate means of assessing in vivo hemolysis, similar studies in humans with either a bile fistula or indwelling T-tubes in the common duct could not be found in the literature. However, assuming that the normal daily output of bilirubin is at least 50 per cent derived from the destruction of $\frac{1}{120}$ of the total red cell mass, or approximately 40,000 red cells per cubic millimeter, it is certain that enough in vivo hemolysis to produce clinical evidence, namely fever or chill, would be reflected in marked elevations of the total bilirubin output. This should be a very sensitive index of hemolysis. No significant elevation of the total or fractional bilirubin output occurred in any of our patients following intravenous fat emulsion. This is taken as evidence that no significant in vivo hemolysis occurred in these subjects.

It should be noted that three patients developed fever of 101.6° F. or above rectally, and two patients had a chill. In none of these individuals did an increase of bilirubin in the bile occur. Conversely, the two patients in whom slight increases did occur did not suffer any adverse reaction whatsoever. Two patients experienced back pain, which is another well-recognized reaction to intravenous fat, but this reaction likewise was not reflected in the quantity of bilirubin excreted. Therefore, it would appear that explanations other than hemolysis must be sought to explain the various reactions produced by parenteral fat emulsions.

CONCLUSIONS

1. Febrile responses to intravenous fat emulsions in human patients are frequent enough to preclude its general usefulness. It has been suggested by others that the cause of the thermogenic response is in vivo hemolysis.

2. Eleven patients with indwelling T-tubes in the common duct were infused with one 600 ml. bottle of a 15 per cent fat emulsion.

3. Quantitative analysis of the bile recovered from the T-tube for bilirubin revealed no significant changes in output of bilirubin after the infusion of fat as compared to control samples.

4. There was no evidence of in vivo hemolysis as a complication of intravenous fat therapy in this group of patients. Since three of the patients developed fever, it is suggested that causes other than hemolysis must be sought to explain the thermogenic response.

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THE PREPARATION AND USE OF ANHYDROUS FAT EMULSIONS FOR INTRAVENOUS FEEDING AND METABOLIC EXPERIMENTS

D. B. Zilversmit, Ph.D., N. K. Salky, M.D.,* M. L. Trumbull, M.D.,** and E. L. McCandless, Ph.D.
Memphis, Tenn.

ATTEMPTS to make a high-calorie, nontoxic fluid for complete intravenous alimentation of patients have brought forth a number of emulsions containing fats in a finely dispersed state.[1-3a] Even though some of these emulsions containing phosphatide emulsifier have proved usable in the clinic, lack of physical and chemical stability has prevented their production in commerical quantities.[3b] Apart from these difficulties the preparation of fat emulsions requires complex machinery which makes it difficult to obtain small quantities of emulsions for metabolic experiments with radioactive lipids. It was particularly this latter problem which required a fundamentally different method of preparing fat emulsions. Since the chemical instability of aqueous emulsions was probably the result of hydrolysis of the glyceride components† of the emulsion, we searched for a method of preparing a prehomogenized anhydrous mixture which could be easily converted to an aqueous fat emulsion of the desired strength by the addition of water. This paper describes the preparation of such a mixture and its use in animals.

MATERIALS AND METHODS

Ten grams of alcohol-soluble soybean phosphatide‡ was suspended in 100 ml. of glycerin (U.S.P. or analytical reagent grade) by vigorous agitation for several minutes in a Waring Blendor equipped with a powerstat. When the phosphatide appeared to be entirely "dissolved," 100 ml. of coconut oil (melting point 76° F.)§ was added, while the stirring was continued until a creamy homogeneous mixture of honeylike consistency was obtained. This took about 2 to 3 minutes. This mixture, known hereafter as the anhydrous base, was stored in a closed container and used to make fat emulsions of 10 to 20 per cent fat content by diluting the base with the appropriate amount of water or 5 per cent glucose. The resulting emulsions were grossly and microscopically indistinguishable from similar emulsions without glycerin which were made with a hydropulse homogenizer at 3,000 pounds of pressure. Quantities of anhydrous base as small as one-tenth the above amounts were prepared in a semimicro steel Waring Blendor cup.

From the Division of Physiology, University of Tennessee, Memphis.
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*U.S. Public Health Service Medical Student Part-Time Research Fellow.
**From the Department of Pathology, Baptist Memorial Hospital, Memphis, Tenn.
†Phosphatides and triglycerides.
‡Alcohol-soluble fraction of soybean phosphatides donated by Glidden Co., Chicago, Ill.
§Coconut oil donated by Humko, Memphis, Tenn.

RESULTS

Physical and Chemical Properties of the Anhydrous Base.—The homogenized base described above is a very thick or syruplike fluid which mixes with water in all proportions. Since most cells are permeable to glycerol, which consequently does not exert its full osmotic pressure across such membranes,[4] the anhydrous base was always diluted with 5 per cent glucose before use. The resulting emulsion contained extremely fine particles, the majority of which were below the visible range of the microscope. Dark-field examination showed that the emulsion contained 2,000 times as many visible particles as could be seen by bright-field microscopy under the same magnification. An occasional 3 μ particle was seen in a high power field of 370 μ diameter. Very rarely an anhydrous base would produce emulsions containing larger particles and was judged unsatisfactory for use.

During the first few animal experiments, little attention was paid to the sterility of the emulsion. However, cultures made from anhydrous base were usually free of pathogenic organisms. This may be the result of the well-known germicidal action of concentrated glycerin solutions.[5] In later experiments all ingredients except the phosphatide were autoclaved before mixing in a sterile Waring Blendor. The anhydrous base itself was not autoclaved since it did not appear to be stable at high temperatures. Later work* indicated that lowering the lecithin content to one-half the original amount improved the heat stability of the pre-mix sufficiently to allow autoclaving. No animal experiments with the autoclaved base have as yet been performed.

Theoretical considerations would predict that the chemical stability of the anhydrous base should be greater than that of anhydrous emulsion, but few data are as yet available. One measure of chemical degradation is the free fatty acid content of the emulsion. We thus compared the free fatty acids per milliliter of 10 per cent oil emulsion freshly made from anhydrous base of different ages with the free fatty acid content of aged aqueous emulsions made of the same ingredients with a high-pressure homogenizer. Both pre-mix and emulsions were stored at room temperature during these aging tests. The free fatty acids were determined by petroleum ether extraction of the emulsion after addition of NaCl, evaporation of the ether, and titration of hot alcoholic solutions of the fat with 0.01 NaOH aqueous. The results in Table I show that although the fatty acid contents of both emulsions were similar when fresh, nearly ten times as much fatty acid was liberated in the aqueous as in the glycerin medium.

Another indication of the physical stability of the anhydrous base is the fact that base made more than a year ago and stored at room temperature gave emulsions of the same microscopic appearance as before storage. Such studies will, however, have to be continued for longer periods of time in order to indicate whether the anhydrous base is more stable than aqueous emulsions.

*Performed by Dr. E. Van Handel.

TABLE I. FREE FATTY ACID CONTENT OF ANHYDROUS BASE AND AQUEOUS EMULSIONS OF DIFFERENT AGES

ANHYDROUS BASE		AQUEOUS EMULSION	
AGE (MONTHS)	MEQ. PER ML. OF 10% OIL EMULSION	AGE (MONTHS)	MEQ. PER ML. OF 10% OIL EMULSION
0	0.0023	0	0.0038
7	0.0037	6	0.023
8	0.0032	8	0.034
10	0.0041	9	0.052
11	0.0058		

TABLE II. RESULTS OF DAILY INTRAVENOUS ADMINISTRATION OF FAT EMULSIONS

DOG	WT. (KG.)	GLYCERIN (ML./KG.)	OIL (ML./KG.)	DAYS ADMINISTERED	ML. ANHYDROUS BASE PER 100 ML. OF EMULSION	CAL. PER KG. PER DAY	REMARKS
GA	9.5	3	3	9	20	45	No ill effects
GC	9.1	3.3	3.3	21	20	50	No ill effects
GD	8.2	3.7	3.7	21	35	50	
GE	10.5	2.3	4.6	21	20	50	
GH	13.6	5.1	5.1	21	30	75	
GG	8.0	5.8	5.8	17	40	75	Convulsions on the last
GI	10.8	5.4	5.4	11	40	75	day
GL	9.1	5.2	5.2	19	30	75	
		4.6	4.6	10†	20		
GN	9.1	4.0		42	15*		No ill effects
GO	9.5	2.6		25	10*		
GJ	9.5	5.4		20	20*		Convulsions on 20th day
		2.6		42†	10*		No ill effects
GK	8.8	5.3		12	20*		Convulsions on 12th day
Acute	14.5	8.3	8.3	1	40		Convulsions at the end
Acute	9.5	7.4		1	20*		of injection
Acute	9.5	7.4		1	20*		

*Milliliters glycerin per 100 ml. of aqueous 5% dextrose.
†After a week's rest period.

Animal Experiments.—To test the physiologic effects of intravenous administration of fat emulsion made from the anhydrous base, we administered the freshly reconstituted emulsion intravenously to the series of mongrel dogs listed in Table II. Each dog received daily a quantity of base diluted with 2.5 to 4 volumes of 5 per cent glucose at a rate of about 300 ml. of emulsion per hour and a total volume of 150 to 470 ml. In order to calculate the number of calories that each animal received parenterally we assumed that the fat, glucose, and glycerol were completely utilized and furnished the following number of calories: fat 9 cal. per gram, phosphatide 8 cal. per gram, glucose and glycerin 4 cal. per gram each. The total quantity of emulsion administered per day furnished 50 to 75 calories per kilogram of body weight.

Since the intravenous fluid contained no amino nitrogen or electrolytes, the animals were allowed free access to Purina Laboratory Chow. Table II lists: daily doses of glycerin and coconut oil, number of consecutive daily injections, concentrations of emulsion, and number of calories calculated as above. The animals have been divided into 5 groups according to their treatment and clinical findings. In addition to daily food consumption, water intake, urine output,

and temperature response, we determined at intervals the hematocrit, and the protein, sediment, pH, and hemoglobin of the urine. Most animals were autopsied 24 hours after the last infusion and the tissues examined microscopically. Furthermore, some animals were injected with one dose of emulsion under anesthesia while blood pressure and electrocardiograms were continuously recorded. Apart from the findings listed in Table II, all laboratory tests did not show any significant differences from control or normal values.

The physical condition and appearance of the animals during the experimental period were excellent. Most animals exhibited good appetites, although their food intake was somewhat below normal. Nearly all animals gained weight. The most noticeable abnormality was the increased water intake and urine output, as has been previously observed.[5] Some animals drank 1 to 2 liters of water per day and were particularly thirsty at the end of an infusion. Since the glycerol exerts, temporarily at least, a considerable osmotic pressure, one might be able to decrease the osmotic effects of the solution by altering the total solute content and thereby restore a more nearly normal water turnover.

The first five animals of Table II received considerable amounts of fat emulsion over a three-week period without ill effects. At the end of the three-week period the experiments were terminated. None of the animals showed any changes in blood or urine constituents and all appeared normal.

The primary pathologic findings were seen in spleens of normal size which showed deposition of fatty material positive for Sudan IV stain. This was deposited as large globules apparently lying free in the red pulp (Dogs GA, GH), as phagocytosed fat in histiocytes (Dog GC), or as traces of fat in small granulomas (Dogs GD, GE). The degree of these changes was scored as moderate to marked in each instance. In addition, three dogs (GD, GE, and GH) had slight to moderate hematopoiesis and hemosiderosis (iron confirmed in the one showing the greatest amount). The only other changes were seen without regularity in the lungs and liver and consisted of mild phagocytosis of fat and iron by Kupffer cells, free fat in pulmonary capillaries, and mild pulmonary edema. No changes were noted in the brain, heart, pancreas, lymph nodes, kidneys, adrenals, and gastrointestinal tract. Bone marrow was not examined.

The next three animals received an emulsion of 30 to 40 per cent anhydrous base at a rate of about 300 ml. per hour and delivering 75 cal. per kilogram. During the first week no abnormalities were noted but in the second and third weeks during the infusions the animals grew restless, developed tremors, and finally went into convulsions. Dog GL convulsed for the first time on the nineteenth day. After a week's rest, infusions were continued at a somewhat lower concentration but convulsions occurred again in 10 days. Apparently this dog was somewhat more sensitive than Dog GH who received the same dose up to the time of sacrifice (21 days) without ill effects. Dogs GG and GI were autopsied and their spleens showed a moderate amount of phagocytosed fat and greater amounts of hematopoiesis and phagocytosed iron; their livers had slight to abundant iron in the Kupffer cells, and the one lung examined for fat exhibited arteries and capillaries filled with homogeneous sudanophilic material

(these animals were autopsied within two hours of the last injection). In an effort to find whether the convulsions were caused by the oil or the aqueous phase of the emulsion, we injected 4 dogs with different doses of glycerin in 5 per cent dextrose. Table II shows that dogs apparently are able to tolerate intravenous glycerin up to 4 ml. per kilogram given in one hour but that at doses of 5 ml. per kilogram or above, convulsions develop in time. Dog GK was autopsied, and except for slight pulmonary congestion, its organs showed no microscopic changes. The last three dogs in Table II show the amount of glycerin required to produce convulsions on the first day of the experiment. Apparently 7 to 8 ml. per kilogram of body weight given intravenously over a period of 30 to 60 min. produced convulsions whether the glycerin was given as an aqueous solution or as part of a fat emulsion. The substitution of analytical reagent grade glycerin for U.S.P. glycerin did not alter its toxicity.

These experiments show the toxicity of glycerin when given in large quantities, but do not indicate the mechanism of action. In a few experiments, serum Ca and Mg were found to be within normal limits, so that the convulsions were not produced by lack of these ions. During convulsions the rectal temperature increased to 104 to 110° F. Sedation of convulsing animals with Nembutal and application of cold cloths stopped the convulsions and depressed the fever; dogs treated in this manner appeared entirely normal the following day. Apparently large doses of glycerin increased the excitability of the central nervous system or the muscles.[6] To date about 100 liters of the reconstituted emulsion have been administered* to 35 patients. These findings will be reported elsewhere.

DISCUSSION

The preparation of a substantially anhydrous fat emulsion concentrate might have advantages if the resulting aqueous emulsion were shown to be free from toxic effects. In these studies we chose to make a fat emulsion from coconut oil and soybean phosphatides, components which had already been successfully used by others.[2] In addition, a procedure was developed which allowed the preparation of emulsions in large or small quantities in a form in which chemical changes should be minimal. It was found that the use of glycerin promoted the dispersion of oil by a Waring Blendor and obviated the use of high-pressure equipment. Furthermore, the incorporation of glycerin allowed storage of a substantially water-free base until the time of dilution with an aqueous medium just prior to use. A different type of "instant" emulsion was recently reported by Shafiroff and Mulholland.[7] For some of our studies we prepared as much as 6 liters of anhydrous base in one batch with an Eppenbach colloid mill and homomixer.† The base was dispensed in 200 ml. aliquots into 1 liter bottles. Just prior to the infusion, 800 ml. 5 per cent sterile glucose was added, and after a few inversions of the bottle the emulsion was ready for use.

Since the final emulsion contained 10 per cent glycerin, one should be aware of the potentially toxic effects of this material. Even though glycerin has been widely used in the pharmaceutical industry and the toxicology of this material

*Conducted by Dr. E. H. Storer, Department of Surgery, University of Tennessee Medical School.

†Obtained from Admiral Tool Co., New York City.

has been studied in various species,[5, 6] little, if any, published data are available on the effects of dilute glycerin solutions administered intravenously. In view of this paucity of data, our own findings of the toxicity of intravenous glycerin cannot be directly compared to work of other investigators. Deichmann,[6] who reviewed the pharmacology of glycerin, quotes that 8 to 10 Gm. per kilogram glycerin given subcutaneously to dogs was lethal in 24 hours, whereas 14 Gm. per kilogram by the same route proved lethal in 3 to 4 hours. This agrees fairly well with our observations that at a dose level of 5 ml. per kilogram (6 Gm. per kilogram) given by slow intravenous drip, tremors and convulsions occurred and ended fatally in animals not treated with Nembutal. However, at daily doses of 4 ml. glycerin per kilogram given as a 10 per cent aqueous solution by the intravenous route, the dogs showed no ill effect as long as three weeks after initiation of the injections.

It appears that although the emulsion collected to some extent in the spleen, the fat must be relatively quickly metabolized, for there was no apparent increase in the size of the spleens in dogs receiving daily injections for twenty-one days. The hematopoiesis in spleen and hemosiderosis in spleen and liver suggest the possibility of a hemolytic effect on blood, but the hematocrit and urine hemoglobin studies do not support this interpretation. Aside from this latter finding, the morphologic changes in the dogs reflect no significant toxic effects from the emulsion.

SUMMARY

By the use of glycerin, oil, and phosphatide, a substantially anhydrous syrup was prepared which upon manual shaking with 5 per cent aqueous dextrose produced a very finely dispersed fat emulsion suitable for intravenous injection. The anhydrous base appeared to be physically stable and prevented to a large extent the hydrolysis of glycerides which normally occurs in aqueous media. Daily injections of reconstituted fat emulsions furnishing 60 cal. per kilogram per day for three weeks were well tolerated by dogs. The toxic limits of intravenous glycerin in dogs were determined.

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PHENOXYMETHYL PENICILLIN (V): PHARMACOLOGIC OBSERVATIONS

PAUL A. BUNN, M.D.
SYRACUSE, N. Y.

WITH THE TECHNICAL ASSISTANCE OF CHARLOTTE MORSEBURG, R.N., BARBARA PALMERI, A.B., AND LEONARD CANARILE, B.S.

THERE is a significant discrepancy between blood concentrations of penicillin following oral and parenteral routes.[1, 2] Generally from two- to fourfold more penicillin given orally is needed to produce levels approximating those following an intramuscular dose. Part of the defect in absorption from the intestinal tract is due to destruction of penicillin in acid contents of the stomach. A type of penicillin which could pass through the stomach in an insoluble, and thus indestructible, state and yet become soluble and thus more absorbable in the duodenum would have a singular advantage.

Phenoxymethyl penicillin (penicillin V), a new type of penicillin, is a free acid, is stable in highly acid medium, is insoluble in acid stomach fluid, and converts easily into a soluble alkaline salt at the usual pH of duodenal contents.[3-8] These physical properties suggest that it might be a suitable preparation for oral use in humans, and a study was, therefore, designed to compare its pharmacologic properties with those of penicillin G. This report describes blood levels and urine excretion of penicillin V following its ingestion by normal adult subjects and these are compared with four other preparations of penicillin G, all given orally.

METHODS

Subjects.—Nine normal young adults were utilized in a cross-over study of blood levels. Each of the 9 individuals was given a standard single oral dose of 400,000 units, and on alternate successive days he received 5 different preparations of penicillin, thus serving as his own control. Seven other normal adults received the same dosage of two or three of the same five preparations of penicillin. There were, thus, from 13 to 16 subjects who received each of the five preparations. Bloods were drawn before, and at one, three, and six hours after the dose. The subjects ingested the penicillin one hour postprandial.

Penicillin.—The five penicillin preparations were tablets or capsules of phenoxymethyl penicillin (V),* buffered tablets of benzyl penicillin (G),† buffered tablets of benzyl penicillin with 0.5 Gm. probenecid added,‡ buffered tables of dibenzylethylene diamine

From the Department of Medicine, State University of New York, Upstate Medical Center at Syracuse, N. Y.

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dipenicillin G (benzathine, DBED, Bicillin†), and a liquid suspension of 1-N-methyl-1, 2 diphenyl-2-hydroxyethylamine penicillin G (1-ephenemine penicillin G or Baz).‡ The choice of the single dose was arbitrary. No special carrying agent for any of the products was included, the tablets for the most part were buffered with citrate.

Measurement of Blood and Urine Levels.—Standard plate techniques, with use of *Sarcina lutea,* as the test organism and penassay seed agar as the base medium, were performed. A standard curve of penicillin's activity was measured daily and the sensitivity of the organism to G and V both recorded. Penicillin G standard was 1,595 units per milligram; that of V 1,695 units per milligram. Final serum concentrations were expressed in units rather than micrograms mostly for convenience in interpretation. End points were read as the clear zone which approximated that corresponding to the 0.1 unit per milliliter standard ring. Levels in serum after each penicillin were uniformly calculated from the G standard and only one test organism was used.

RESULTS

At One Hour.—Peak serum concentrations with 4 of the 5 preparations are observed at one hour following an oral dose of 400,000 units (Fig. 1 and Table I). At that time there is variation among the 16 subjects and 5 drugs, but in general the highest individual peak (2.4 units per milliliter), the highest average level (1.09 units per milliliter), and the best of the low levels (0.31 unit per milliliter), occur in subjects receiving penicillin with

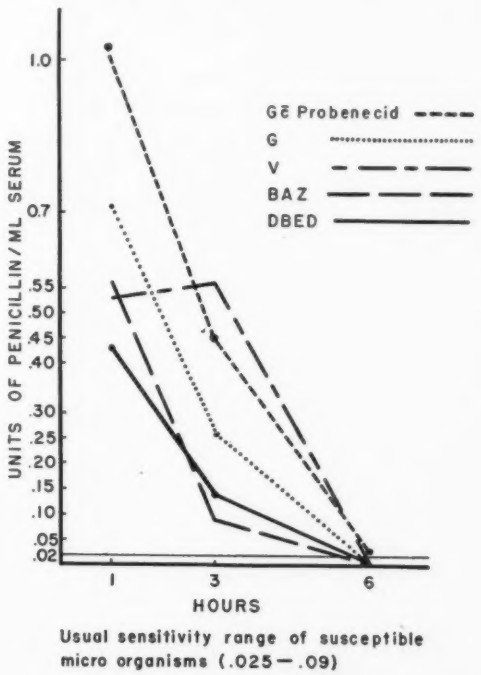

Fig. 1.—Penicillemia following oral dose of 400,000 units of five preparations of penicillin given one hour postprandial.

probenecid. Contrariwise, one of the lowest levels (0.02) and the lowest average (0.43 unit per milliliter) are observed after a dose of DBED (Bicillin). Levels attained following the dose of G, Baz, and V are intermediary. Con-

TABLE I. CROSS-OVER STUDY OF PENICILLEMIA IN NORMAL SUBJECTS INGESTING ONE HOUR POSTPRANDIAL 400,000 UNITS OF EACH OF FIVE PREPARATIONS

PRODUCT	NO. SUBJECTS	UNITS PENICILLIN/ML. SERUM							
		AT ZERO HOUR (BEFORE DOSE)	AT 1 HOUR		AT 3 HOURS		AT 6 HOURS	
			RANGE	AV.	RANGE	AV.	RANGE	AV.
G	13	0	0.05-1.75 (1)	0.71	0.07-0.66 (1)	0.21	0.05 (12)	<0.02
V	13	0	0.05-1.8 (1)	0.53	0.06-2.2*	0.46	0.02-0.03 (11)	<0.02
G with probenecid (1 Gm.)	13	0	0.31-2.4 (1)	1.09	0.06-1.0 (1)	0.43	0.03-0.065 (4)	>0.03
DBED (Bicillin)	13	0	0.02-1.25 (3)	0.43	0.04-0.40 (1)	0.15	0.02-0.11 (6)	<0.02
Baz (1-ephenamine)	16	0	0.03-1.8	0.56	0.02-0.22	0.09	0.04-0.056 (14)	<0.02

( ) Number of samples with no detectable level.

*One subject had 2.2 units per milliliter. Without this single high level, average concentrations would be 0.42 micon per milliliter.

centrations of V range from 0.05 to 1.8 units per milliliter with an average of 0.71 unit per milliliter; Baz levels extend from 0.03 unit per milliliter to 1.8 units and its average is 0.56 unit per milliliter.

It is noted that individual subjects have reasonably constant levels after each penicillin preparation; often if high with one preparation, the level is high with the other forms. For example, the individual attaining the highest level observed following the dose of probenecid and penicillin (2.4 units per milliliter) has the second highest level after Baz and V and the third highest level after G.

At the first hour penicillin G in tablets had higher levels than penicillin V. The difference is not of statistical significance (Fig. 1). When blood levels of the nine subjects who received all 5 preparations are analyzed statistically by a simplified "t" test, concentrations of DBED penicillin are significantly lower than Baz and probenecid.* Because of the variables and the few numbers, it cannot be stated that probenecid levels are significantly higher than those of either G or V.

At the Third Hour.—At 3 hours the relationships change (Table I and Fig. 1). The highest individual level (2.2 units per milliliter), the highest average (0.46 unit per milliliter), and the highest low level (0.06 unit per milliliter) are noted after penicillin V. With this preparation, peak concentration in serum occurs between the first and third hour and in this respect is different from the other 4 materials whose peak levels occur at or near the 60-minute point. Penicillin with probenecid has the second highest level, its average is 0.43 unit per milliliter, the lowest concentration is 0.06 unit per milliliter and the highest is 1.0 unit. Penicillin G is third, its average level is 0.21 unit per milliliter. DBED produces slightly higher levels than Baz. Again it is observed that each subject is quite consistent in his ability to absorb the various penicillins and to attain nearly similar curves of blood concentrations with each preparation. For example, of the 3 specimens which had no detectable level at 3 hours, all came from the same subject (Table I).

At Six Hours.—Blood serum levels of penicillin at 6 hours following a dose of 400,000 units of each of 5 preparations are too low and too infrequent to compare accurately one with the others (Table I and Fig. 1). In the case of penicillin with probenecid, 8 of the 13 subjects have measurable levels ranging from 0.03 to 0.063 unit per milliliter. Seven of 13 subjects have levels of DBED ranging from 0.02 to 0.05 unit. With the other 3 preparations no more than 2 of the 13 subjects have detectable levels and none is higher than 0.04 unit.

Urine Excretion.—Urine excretion after penicillin V was measured in 10 of the subjects. From 71,000 to 170,000 units (18 to 42 per cent) were recovered daily. From 10 to 33 per cent of a single dose of G was found in the urine during the 24 hours after a dose.

*Statistical analyses of the paired sera were done by Dr. M. A. Brumbaugh, and Miss Geraldine F. Fellers, Syracuse, N. Y., and we are indebted to them for their interpretations.

DISCUSSION

It is our opinion that when blood levels after the administration of a number of penicillin preparations are compared, similar techniques of measurement are desirable. It does not seem sensible to use separate testing microorganisms because various preparations may have slightly different degrees of activity upon a single strain or species of bacteria. As is emphasized by Finland and Jones,[5] there is a significant difference in minimal inhibiting concentrations of G and V when group A beta hemolytic streptococci (str. 98) and micrococci pyogenous, var. aureus (str. 209 p) are used. Similarly the minimal detectable blood concentrations of the two drugs are different according to the organism used for testing each of them. For reasons of simplicity, consequently the cup-plate method, utilizing *Sarcina lutea* as the test organism, was used throughout this study, and the final serum levels following all 5 preparations were measured against a G standard and recorded as a single end point. In this way the antibacterial standard is constant.

From the work reported by Finland and associates,[5, 6] by Welch and his associates,[4, 7] and by others,[3, 8] it is evident that the ingestion of penicillin V results in higher and more prolonged blood levels than occurs after penicillin G. The observations recorded here also indicate that satisfactory, prolonged and slightly higher levels after oral administration of V do indeed occur. This is particularly so after the first hour and before the sixth. Further, the amount of a single dose absorbed, as measured by determining that excreted in the urine over the following 24 hours, is somewhat in excess of that absorbed after a dose of penicillin G (average about 25 per cent versus about 17 per cent). Although others have claimed that blood levels are obtained more dependably following the administration of V than after G,[3, 8] our data do not show this, for both preparations are equally reliable in producing detectable concentrations in serum. Thus, there is a superiority of V over 3 of the other preparations, but the difference is not particularly striking, at least as determined by measuring blood levels and urinary excretion and when the levels are subject to statistical analyses.

There still exists also, with V's oral use, a marked deficiency of absorption when compared to an intramuscular dose of G. For example, from 60 to 90 per cent of an intramuscular dose is recovered in urine after parenteral administration;[1] at best only 40 per cent of an oral dose of V is recoverable. This is not unexpected. Although penicillin G is destroyed in acid, only a small proportion of an ingested dose is actually lost in the stomach, perhaps no more than 10 to 15 per cent of a single dose.[1] The major loss of penicillin in the intestinal tract, which is approximately two-thirds of any dose, is due to poor and incomplete absorption from the duodenum (very little is absorbed below this area). Loss in the stomach would be avoided by the use of penicillin V, for it is not destroyed in acid, but there is no information to suggest that V is absorbed faster or to a greater degree from the duodenum or from the remainder of the G.I. tract than is G. It is, therefore, hardly likely that it can produce blood levels comparable to those attained after a parenteral

dose of either G or V. The superiority of oral V over G is due mainly, perhaps solely, to its complete passage through the stomach without destruction, accounting for a saving, at most, of from 10 to 15 per cent of every dose. This presumption is more or less borne out by the minor differences in blood levels between the two observed in this study, and in the amounts found in the urine. However, as levels following an oral dose are not depreciated by food in the stomach,[5, 6] it can be given without regard to meals. This is, in some respects, the singular advantage of V, for it is certainly not the case with a prescription of G.[9]

It must be emphasized that the wide variability of levels with all preparations at the one- and three-hour periods after a dose (Table I) indicates clearly that significant changes in clinical dosage are not warranted with any preparation. For example, as is shown, at least one subject fails to develop detectable levels after 4 of the 5 preparations studied. At both periods the levels which are measurable range from very low to unusually high ones. Although individuals are reasonably consistent in absorbing the various preparations, there is, among the whole group, such wide variations in levels that predictability with any one preparation is not possible. (The ranges cannot be analyzed statistically.) The statement about clinical prescription thus becomes more than speculation or presumption.

Finally, these data point up one important fact. It is clearly evident that no one of the preparations of penicillin studied yields serum levels high enough or prolonged for a sufficiently longer period in the effective range of concentration to permit a change from the intramuscular to the oral route for the management of any human infection not now treatable by the oral route. Presently recommended dosage schedules for oral prescription of penicillin G also should not be appreciably altered by the substitution of either penicillin V or penicillin G with probenecid. All oral preparations probably should be prescribed in approximately similar fashion.

SUMMARY

When compared with a buffered penicillin G tablet, a rapidly disintegrating capsule or tablet of penicillin V resulted in higher blood levels 3 hours after a single dose of 400,000 units given to healthy adults who had eaten 60 to 90 minutes before the dose. At one and six hours after the dose, blood concentrations were essentially the same.

Somewhat more penicillin V is recoverable in urine than occurs after a dose of penicillin G.

Neither preparation of penicillin produced serum levels comparable to those attained and maintained after a dose of penicillin G with 1 Gm. of probenecid, particularly so at the first and sixth hours.

These findings suggest that penicillin V is an excellent preparation for oral administration and can be used for therapy of human infections caused by penicillin-susceptible organisms. They do not suggest that penicillin V can be prescribed in doses appreciably less than those now commonly recommended before penicillin G tablets.

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METABOLISM OF CITROVORUM FACTOR AND FOLIC ACID IN GOUT; URINARY EXCRETION STUDIES

WILLIAM W. FALOON, M.D., AND SHIRLEY DILG, M.S.
SYRACUSE, N. Y.

WITH THE TECHNICAL ASSISTANCE OF
KATHLEEN COLLINS, B.S.

USING N^{15}-labeled glycine, Benedict and associates[1] have found that certain patients with gout have an increased rate of uric acid formation. Similarly, Spilman[2] has reported that C^{14} administered as formate appears in higher concentrations and more rapidly in the urine uric acid of gouty persons than in normals. These workers have theorized that hyperuricemia in these individuals may be a result of increased urate formation by nonnucleoprotein pathways. The role of citrovorum factor (CF) or a closely related compound in purine and uric acid formation has been suggested in the demonstrations by Buchanan and Wilson[3] and by Greenberg[4] that such compounds may act as transformylating agents in the formation of purines. These studies indicate the possibility that the metabolism of the folic acid (FA) and CF group of compounds may be disturbed in gouty patients. Thus, the demand for FA and particularly for CF may be increased in order to carry out transformylation in accelerated purine synthesis. Either increased conversion of pteroylglutamic acid (PGA)* to CF or an increased utilization of the latter might be observed in these individuals. Therefore, urinary excretion studies of FA and CF following the administration of these factors to patients with gout have been carried out. In addition, the effect of ascorbic acid which has been shown to stimulate conversion of PGA to CF[5] has been investigated. After certain abnormalities in CF metabolism in gout were observed in the results to be presented, the investigation was extended to include the effects of salicylates, phenylbutazone, probenecid, and colchicine upon urinary CF excretion.

MATERIALS AND METHOD:

Nine studies of urinary FA and CF excretion were carried out in eight patients with clinical and laboratory evidence of gout but in whom there was no overt evidence of associated renal disease. The pertinent data on these patients are shown in Table I. Similar studies were carried out in four hospitalized, nongouty patients whose diagnoses and pertinent data are also shown in Table I and in eight male and two female, healthy, normal

From the Department of Medicine, State University of New York Upstate Medical Center, the University Hospital of the Good Shepherd, and the Veterans Administration Hospital, Syracuse, N. Y.

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*PGA denotes pteroylglutamic acid while FA is used herein to denote all substances stimulating growth of *Streptococcus fecalis*.

TABLE I

PATIENT	AGE	TYPE OF GOUT	NPN (MG./100 ML.)	SERUM URIC ACID AT TIME OF STUDY (MG./100 ML.)	ASSOCIATED DIAGNOSIS AND COMMENT	DIET DURING STUDY
M. R. I	40	Chronic	25	5.5 (After therapy)	Received colchicine and phenylbutazone prior to PGA	Regular hospital
M. R. II		Second study	28	6.6	Restudied 8 mo. after first study. Received probenecid until 3 days before study	Mod. restricted purine
M. D.	77	Chronic tophaceous	29	8.2	Angina pectoris, osteoarthritis. Salicylate therapy until 4 days before study	Mod. restricted purine
W. K.	61	Intermittent acute	23	6.4	Lobar pneumonia, convalescent. Received aqueous penicillin and streptomycin until 2 days before study	Mod. restricted purine
F. B.	72	Intermittent acute	26	7.1	No recent medication	Mod. restricted purine
J. C.	56	Intermittent acute	26	6.7	Coronary occlusion, convalescent. Received papaverine and Dicumarol prior to study	Mod. restricted purine
J. A.	62	Intermittent acute	14*	7.2	Pulmonary emphysema and arteriosclerotic heart disease. Received pyribenzamine, digitoxin, and SSKI during study	Mod. restricted purine
C. R.	72	Intermittent acute	18*	7.3	Arteriosclerotic heart disease, osteoarthritis. Received phenobarbital, digitoxin, and aminophylline during study	Mod. restricted purine
L. R.	54	Chronic tophaceous	25	10.1	Duodenal ulcer, previous bleeding, and anemia of 7 Gm. hemoglobin. Belladonna during study	Bland diet

HOSPITAL NORMALS

PATIENT	AGE	DIAGNOSIS	COMMENT
R. Ba.	62	Convalescent coronary occlusion	Mod. restricted purine
R. D.	58	Parkinson's disease, hypertension, serological syphilis	Mod. restricted purine
R. R.	57	Multiple sclerosis, pulmonary emphysema	Mod. restricted purine
L. G.	40	Neurosis, cyst of olecranon bursa. Received multiple vitamin capsule until PGA begun and phenobarbital throughout study	General hospital

All gout patients and hospital normals were males. Serum uric acid was less than 5.5 mg./100 ml. in all normals.

*BUN determination.

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laboratory assistants and medical students. Daily urinary excretion of FA, CF, and in some patients, uric acid, was measured. After one or more control days, the subjects were given PGA* in doses of 5 mg. twice daily orally. The gouty subjects received either a moderately restricted purine diet (containing an average of 196 mg. [114 to 260] of purine and 80 to 180 mg. of ascorbic acid daily) or a general hospital diet. One patient was studied while receiving a bland diet for an associated duodenal ulcer. Similar diets were given to the hospital normals and the healthy normals were studied while eating their usual diets with instructions to restrict their intake of sources of ascorbic acid. Similar dietary regimens were followed during the studies in which CF (Leucovorin) was given intramuscularly in doses of 3 mg. daily. This dosage provided approximately 40 to 50 mcg. per kilogram of body weight for all individuals. Three patients with gout were studied during the oral administration of ascorbic acid in doses of 1 Gm. daily while receiving PGA.

Urines were collected under toluene, and at the end of the 24-hour collection, aliquots were alkalinized and frozen for subsequent analysis for FA and CF. Other aliquots were acidified and frozen for uric acid analyses. Fasting blood specimens for uric acid and ascorbic acid were collected during or preceding the PGA period. The serum for uric acid was removed and frozen and the specimens for whole blood ascorbic acid determinations were preserved in trichloracetic acid and refrigerated for subsequent analyses.[6] Uric acid was determined by Archibald's[7] modification of the method of Kern and Stransky.[8] Microbiologic assay of FA which included all growth substances for *Streptococcus fecalis* was done by the method of Lucky and associates[9] and assay of CF was done by the method of Sauberlich and Bauman.[10] Synthetic citrovorum factor (Leucovorin) was used for the CF standard. The standard solution was newly prepared at approximately 30-day intervals and comparative assays carried out with the old and new standards revealed no significant change in the growth curves obtained with each new standard solution. The analyses for folic acid were not corrected for stimulation of growth by the CF present in the specimens. Stimulation of the growth of *Streptococcus fecalis* by 1 gamma of CF was determined to be slightly less than that provided by 1 gamma of PGA.

In the experiments on urinary excretion of CF following an intramuscular administration of Leucovorin, all subjects received injections of the same lot of Leucovorin and this lot was also used as the standard for these urine assays.

RESULTS

I. *CF and FA Excretion Following PGA Administration.*—The average daily urinary excretion of CF during the total PGA period in the nine studies in gout patients was 18.4 gamma, which was significantly lower (P value < 0.01) than the average of 38.1 gamma excreted by normal individuals (Table II). The comparative ranges of values for the various groups of patients are shown in Fig. 1. Although CF excretion in some gouty patients overlap with the results obtained in normals, four of the gout patients excreted an average of less than 13 gamma of CF daily during the administration of PGA. In contrast, the urinary excretion of FA by the gout patients showed no difference from that by healthy normal individuals, whereas the three hospital "normals" in whom urinary FA was measured showed an average excretion of less than half that observed in the other two groups (Fig. 2).

Whole blood ascorbic acid levels were lower in the gout patients than in either the hospital normals or healthy normals (Fig. 3), but no correlation

*BUN determination.

*PGA and CF (Leucovorin) were kindly supplied by Lederle Laboratories, Pearl River, N. Y.

between whole blood ascorbic acid and average CF excretion by individual normal or gouty patients was observed. This lack of correlation between blood ascorbic acid and CF excretion is shown in Fig. 4.

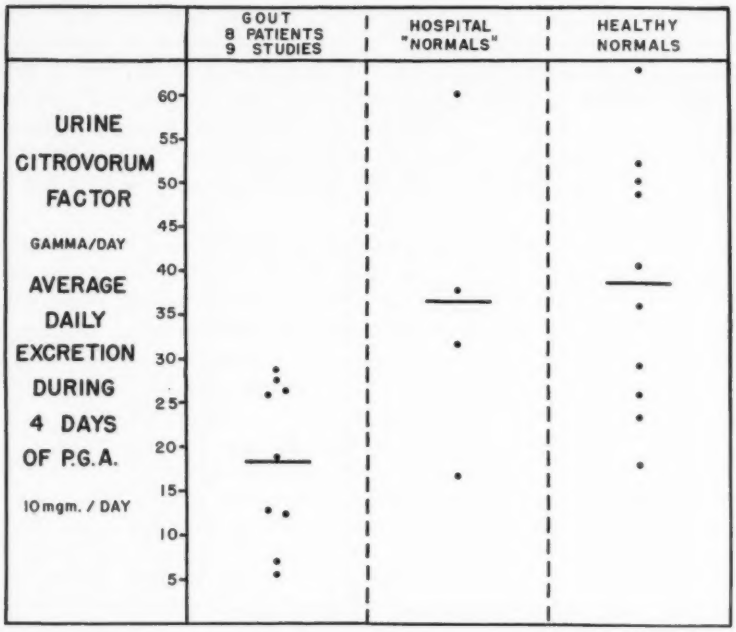

Fig. 1.—Average 24-hour CF excretion during four days of PGA. Horizontal bar indicates average of each group.

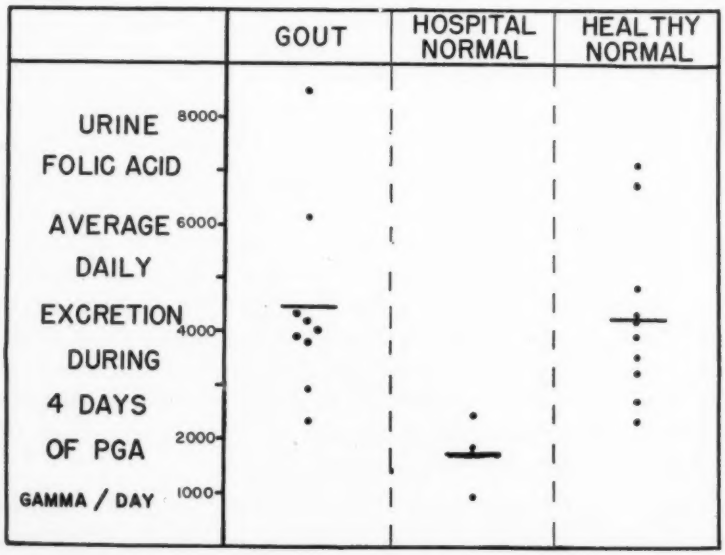

Fig. 2.—Average 24-hour excretion of FA during four days of PGA. Horizontal bar indicates average of each group.

TABLE II. DAILY URINE CF AND FA EXCRETION (GAMMA/DAY) BEFORE AND DURING PGA ADMINISTRATION

GOUT PATIENTS		CONTROL	DAYS OF PGA 1	2	3	4	AVERAGE DAILY EXCRETION
M. R. I	CF	0.5	14	11	13		12.7
	FA	2.5	4,603	15,800	5,120		8,507
	Asc. Acid mg. %						
M. R. II	CF	7.6	22.6	27.2	26.9	28.5	26.3
	FA	8.3	2,105	2,360	3,024	4,180	2,917
	ASc. Acid	0.43					
M. D.	CF	2.8	6.0	3.7	6.6	5.5	5.5
	FA	5.3	5,400	4,160	2,492	5,256	4,327
	Asc. Acid		0.22				
W. K.	CF		11.6	5.7	5.8	29.8	13.2
	FA		1,694	2,793	3,116	1,805	2,352
	Asc. Acid					0.19	
F. B.	CF	1.1	7.0	5.8	8.1	7.3	7.0
	FA		4,000	6,342	7,675	6,557	6,143
	Asc. Acid				0.25		
J. C.	CF	0.9	21	12.7	21.7	54.8	27.6
	FA	4.3	4,560	2,400	4,240	4,530	3,932
	Asc. Acid	0.65					
J. A.	CF	3.3	5.7	18.9	29.6	49	25.9
	FA	3.9	2,660	3,600	4,625	4,660	3,886
	Asc. Acid			1.16			
C. R.	CF	0.8	29	13	23	10	18.8
	FA	2.9	2,650	5,200	5,720	3,350	4,230
	Asc. Acid					0.52	
L. R.	CF	0.3	56	19	23.9	17.1	28.8
	FA	2.2	5,304	4,437	3,498	2.812	4,012
	Asc. Acid					0.46	
Average	CF	2.1	18.9	13.0	17.6	25.2	18.4
	FA	4.2	3,664	5,332	4,435	4,144	4,478
HOSPITAL NORMALS							
R. Ba.	CF	1.0	22	20.4	14.7	9.8	16.7
	FA	2.0	1,736	2,244	2,538	3,316	2,458
	Asc. Acid	0.48					
R. D.	CF	2.3	30.8	43	66.5	100	60.1
	FA	5.8	984	1,075	1,120	606	946
	Asc. Acid	0.67					
R. R.	CF	2.1	26.3	26.5	22.5	50.5	31.6
	FA	2.1	2,230	1,696	1,904	1,710	1,885
	Asc. Acid	0.49					
L. G.	CF	4.7	39.8	32.3	34.2	45	37.8
	FA	0.7					
	Asc. Acid	1.2					
Average of Hospital Normals	CF	2.5	29.7	30.5	34.5	51.3	36.5
	FA	3.3	1,650	1,671	1,854	1,877	1,763
HEALTHY NORMALS							
H. G.	CF	1.9	42.4	38.4	113.8	57	62.9
	FA	1.0	3,388	2,944	6,640	3,976	4,237
	Asc. Acid	1.0					
F. Y.	CF	0.4	34.5	26.6	43.5	40	36.1
	FA	0.7	3,864	5,920	3,480	13,800	6,266
	Asc. Acid	1.3					

TABLE II—CONT'D

HEALTHY NORMALS		CONTROL	DAYS OF PGA 1	2	3	4	AVERAGE DAILY EXCRETION
R. B.	CF	1.0	11	42.6	25.4	27	26
	FA	1.3	4,320	4,014	6,172	2,980	4,371
	Asc. Acid	1.4					
P. G.	CF	1.3	24.6	16	31.9	21	23.4
	FA	1.1	1,640	3,340	3,896	1,989	2,716
	Asc. Acid	0.58					
P. O.*	CF	0.5	2.9	17.8	42.0	9.4	18.0
	FA	0.8	812	3,550	8,700	15,500	7,140
	Asc. Acid	1.0					
C. M.*	CF	0.7	16.7	23	7.2	70.4	29.3
	FA	0.6	3,441	3,128	2,002	805	2,344
	Asc. Acid	0.96					
R. W.	CF	1.7	27.4	56.8	54.9	61.8	50.2
	FA	3.9	4,276	5,051	7,255	5,785	4,841
	Asc. Acid	0.49					
J. B.	CF	1.4	33.8	48.2	65.6	47.5	48.7
	FA	1.5	4,059	4,139	1,592	5,890	3,920
	Asc. Acid	0.62					
M. C.	CF	2.3	21.5	83.5	61.8	45.5	52.3
	FA	1.5	2,789	4,092	3,575	3,710	3,541
	Asc. Acid	1.07					
J. L.	CF	2.3	23.8	44.5	36.8	57.0	40.5
	FA	1.8	1,995	2,314	3,381	5,320	3,252
	Asc. Acid	1.12					
Average of Healthy Normals	CF	1.3	23.8	39.7	48.2	43.8	38.8
	FA	1.4	3,058	3,849	4,369	5,975	4,312
Average of all normals	CF	1.7	25.5	37.1	45.1	45.9	38.1
	FA	1.8	2,731	3,346	3,788	5,029	3,724
9 Gout, Mean	CF	18.4			14 Normals, Mean CF		38.1
	S.D.	9.2				S.D.	15.0
	S.E.	3.1				S.E.	4.0
			p value $<$ 0.01				

*Female.

The average excretion of FA and CF on individual days in gout patients and in the normals on successive days of PGA administration are charted in Fig. 5. There was no significant difference in FA excretion on either the control day or on any of the four days of PGA administration. In contrast, however, the average CF excretion was less in the gout patients than in the normals except on the control day. This difference was most marked on the third, fourth, and fifth days of PGA. On these days the average excretion of CF by gout patients was approximately half that of the normal individuals.

II. *CF Excretion Following Parenteral CF.*—CF (Leucovorin) was given intramuscularly on two successive days to three normals and to three patients with gout who were restudied six months or more after the PGA administration experiments. Urinary excretion of CF by the gouty individuals was less than half ($P < 0.01$) that of the normal individuals (Table III). Similarly, less FA was excreted during the days of CF administration, which would correlate with the fact that the apparent FA activity in the urine was probably largely due to CF. In these studies no difference in the range of ascorbic acid levels in the normals and in the gouty patients was present.

III. *Effects of Administered Ascorbic Acid.*—In three patients the effect of the administration of ascorbic acid for four to six days simultaneously with the administration of PGA was studied. As shown in Table IV the response of these gouty individuals followed the expected pattern with increasing excretion of CF during ascorbic acid administration. In two of the three

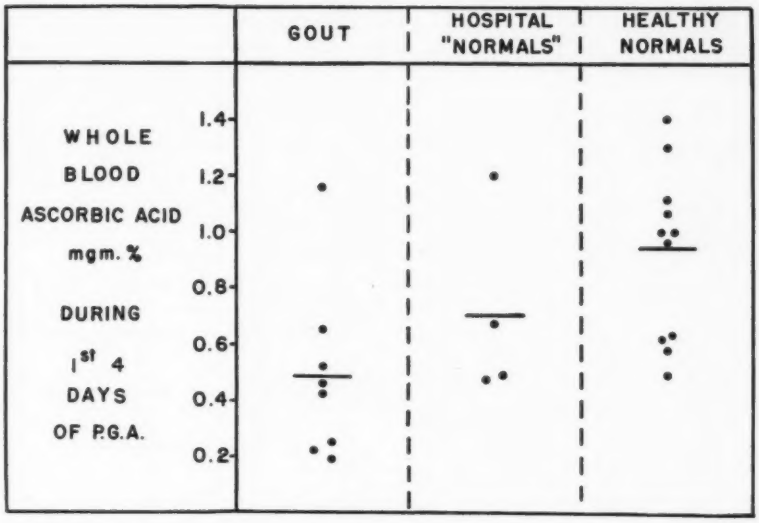

Fig. 3.—Blood ascorbic acid concentration during the PGA period. Horizontal bar indicates average of each group.

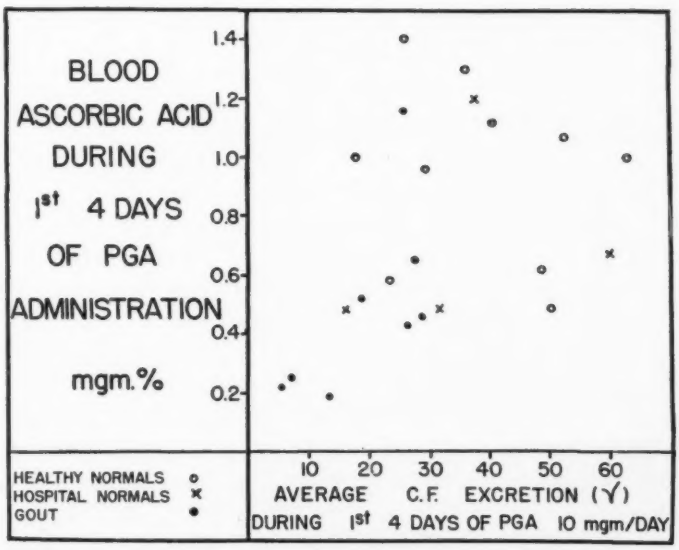

Fig. 4.—Comparison of blood ascorbic acid concentration and average 24-hour CF excretion. No correlation is observed.

patients there was a marked increase in urine excretion of CF while in the third there was a significant rise but to a lesser degree. A typical response to the administration of ascorbic acid is shown in Fig. 6.

TABLE III. URINARY EXCRETION OF CF AND FA FOLLOWING INTRAMUSCULAR CF, 3 MG. DAILY FOR 2 DAYS

NORMALS	DAY	GAMMA/DAY CF	GAMMA/DAY FA	BLOOD ASC. ACID (MG./100 ML.)	GOUT	DAY	CF	FA	BLOOD ASC. ACID (MG./100 ML.)
R. B.	1	549	490	1.2	M. D.	1	8	50	0.31
	2	603	441			2	290	355	
C. M.	1	569	265	0.83	W. K.	1	375	457	1.02
	2	581	605			2	120	300	
P. G.	1	552	398	0.58	F. B.	1	202	302	0.64
	2	422	703			2	153	184	
Average		546	483.6				191.3	274.6	
CF Excretion: S.D. = 64.3 S.E. = 26.2					CF Excretion: S.D. = 129.2 S.E. = 52.7				
p value < 0.01									

TABLE IV. EFFECT OF ASCORBIC ACID ON CF EXCRETION

DAYS	PATIENT M. D. INTAKE PGA (MG.)	ASC. ACID	URINE CF (GAMMA)	PATIENT C. R. INTAKE PGA (MG.)	ASC. ACID	URINE CF	PATIENT J. C. INTAKE PGA (MG.)	ASC. ACID	URINE CF
1	10		6.0	10		29	10		21
2	10		3.7	10		13	10		12.7
3	10		6.6	10		23	10		21.7
4	10		5.5	10		10	10		54.8
5	10	1 Gm.	30.8	10	1 Gm.	14	10		48.4
6	10	1 Gm.	69.6	10	1 Gm.	35	10	1 Gm.	206
7	10	1 Gm.	179	10	1 Gm.	41	10	1 Gm.	212
8	10	1 Gm.	100	10	1 Gm.	54	10	1 Gm.	
9	10		264	10		50	10	1 Gm.	451
10	10		91.6	10		31	10	1 Gm.	579
11							10	1 Gm.	341
12							10		205

IV. *Effect of Therapeutic Agents.*—Two patients received acetylsalicylic acid orally in doses of 4 Gm. daily during the administration of 10 mg. of PGA daily. One of these patients was also given acetylsalicylic acid during a period when no PGA was being given. Despite increased excretion of uric acid no significant effect on CF excretion was observed. The urinary CF excretion rose during the administration of salicylates in two patients but there was no corresponding fall when the salicylate was withdrawn. The rise, therefore, appears to have been due to the increase in CF excretion which might be expected with prolonged administration of PGA.

Phenylbutazone was administered orally in doses of 400 to 800 mg. daily during the PGA period in two patients and in one of these patients this compound was also administered prior to PGA. In a third patient the administration of phenylbutazone followed a period of PGA administration but the two compounds were not given simultaneously. No effect of phenylbutazone on the excretion of CF was demonstrated.

The effect of probenecid in doses of 2 Gm. daily was studied in one patient receiving PGA and in another patient who was not. In the latter individual 3 Gm. of sodium bicarbonate was given orally with the probenecid.

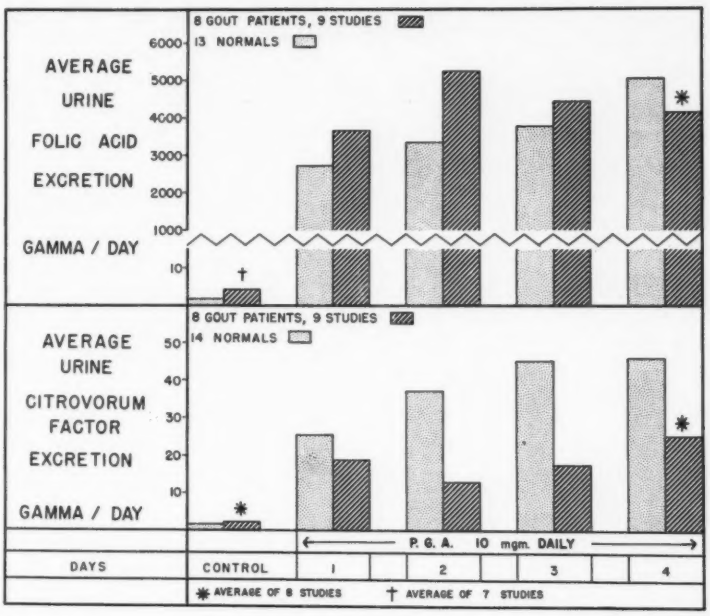

Fig. 5.—Average excretion of FA and CF by gouty and normal groups on individual days of the study. Note marked difference in CF excretion throughout PGA administration.

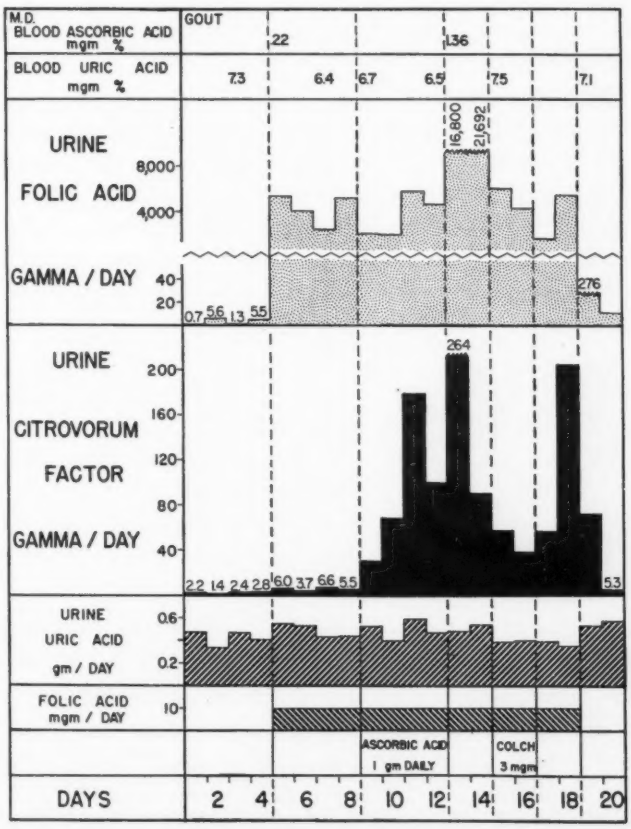

Fig. 6.—Effect of PGA administration upon CF, FA. and uric acid excretion. Stimulation of CF formation by ascorbic acid is apparent but colchicine shows no definite effect. Urine and blood urate concentrations show no changes attributable to the PGA, ascorbic acid, or colchicine. On this chart, folic acid, mg./day, refers to oral PGA.

In neither patient did the probenecid appear to increase the excretion of CF despite a clear-cut uricosuric effect in the one patient in whom urine urate was determined during ingestion of a low purine diet.

Two patients, M. D. and M. R.$_I$, were studied during the administration of of colchicine. In M. D., the drug was administered in doses of 0.6 mg. every 2 hours for 5 doses on each of 2 successive days while concurrently receiving PGA. As can be seen in Fig. 6, this study followed by 2 days the period of ascorbic acid administration. Patient M. R.$_I$ received 1.2 mg. every hour for 10 doses on one day during a period of no PGA supplement. CF excretion on this day was 2 gamma as compared to excretion on the two preceding and two succeeding days of 1.5, 3.0, 1.0, and 2.0 gamma, respectively. Thus colchicine had no apparent effect upon CF excretion under the conditions of either of these studies.

V. *Serum and Urine Urate During PGA.*—In 5 of the gout patients serum and urine uric acid analyses were carried out before and during PGA administration. In none of the patients, all of whom received the moderately restricted purine diet, was there a significant change in either serum urate level or in urate excretion. In addition there was no correlation between urine urate and CF or FA excretion. A typical study of urate in serum and urine during PGA administration is shown in Fig. 6.

VI. *Uric Acid Excretion on Low Purine Diets in Gouty Individuals Previously Studied for CF Excretion.*—Three of the patients previously studied on only moderately restricted purine diets had determinations of uric acid excretion while receiving low purine diets in order to determine the degree of uricosuria existing under these circumstances. As shown in Table V, these three individuals who had low urinary CF excretion during PGA administration did not excrete excessive amounts of urate during ingestion of this diet.

TABLE V. URATE EXCRETION ON LOW PURINE DIET

DAY	MG. PER DAY		
	M. D.	W. K.	F. B.
1	422	177	229
2	435	218	173
3	253	208	319
4	405	165	
5	424	144	

DISCUSSION

Although studies of daily urinary excretion of CF during administration of PGA in doses comparable to ours are infrequent in the literature, Gabuzda and co-workers[11] have reported values of CF excretion following administration of PGA in 10 mg. doses which are quite similar to our data. Welch and associates,[5] using doses of 5 and 50 mg. of PGA orally, have reported that the average urinary excretion per day on such doses was 16 gamma and 164 gamma, respectively. By extrapolation one would expect that their subjects would have excreted approximately 32 gamma daily if they had received 10

mg. doses of PGA. On the basis of this limited evidence it would appear that the range of CF excretion by our normals is comparable to that obtained by other workers.

These studies indicate that CF excretion is decreased in gouty patients under conditions of PGA or CF administration. This observation may be explained by one or more of several mechanisms. These include diminished conversion of PGA to CF, depleted body stores of CF, increased CF utilization, or decreased renal excretion. The low CF excretion following its parenteral administration suggests that the defect is probably not in the conversion of FA to CF. Depleted body stores of CF would appear unlikely in that gout patients did not show progressively increasing CF excretion during the four days of large doses of PGA. In addition, the previous dietary intakes of most of the patients with gout were within normal limits prior to the time of study. The absence of a decreased excretion of CF on the control days by the patients with gout would also suggest that body stores of this vitamin were not depleted.

The diets ingested during the period of study by the two groups, particularly the difference between the diets of the healthy normals and the gouty individuals, may play a role in the observed differences in excretion of CF. Register and Sarett[12] found increased urinary CF excretion during ingestion of high purine diets in studies without PGA supplementation. Thus a lower purine diet such as that given the gout patients might result in lower CF excretion. In our studies three of the hospital normals received restricted purine diets comparable to those ingested by the gout patients. Two of the three individuals fed such diets, however, had urinary CF values which were higher than the highest gout patient. This would indicate that the role of the diet is probably minimal in explaining the decreased CF excretion.

The previously mentioned evidence that some patients with gout have a high rate of purine formation and the demonstration that CF or a related factor is concerned in the synthesis of purines suggest that increased CF utilization may explain our findings. This, however, cannot be definitively proved on the basis of these studies, since observations on the disappearance of CF from plasma and on renal clearance of CF will be necessary to distinguish between tissue utilization and the possibility of a primary renal retention of CF. The demonstration of normal FA excretion by gout patients in these studies indicates, however, that primary renal retention of CF is unlikely in view of the chemical structural similarity between CF and PGA.

In addition to the failure to excrete CF normally following PGA administration, these patients also excreted less CF than normals when CF was administered parenterally. Evidence that the excretion of this factor in our normal individuals is not excessive is available in the studies of Broquist and associates.[13] These workers have reported six-hour urinary excretion data for normal individuals given CF intravenously. The average excretion of 674 gamma in 6 hours approximates the 24-hour urinary excretion in our normal individuals given similar doses intramuscularly. In their experience most

of the urinary excretion following intravenous administration occurs in 6 hours and if the different routes of administration can be compared, urinary CF in our normals is quite similar to their results.

Stimulation of PGA conversion to CF by the administration of ascorbic acid has been well demonstrated in normals[5, 13] and in scorbutic individuals.[4, 11] In our studies, despite the occurrence of lower blood ascorbic acid levels in gout patients as compared to normals, CF excretion did not correlate with ascorbic acid levels. The response to ascorbic acid administration in the gout patients was marked in two patients and significantly increased in the third, although to a lesser degree. Although no comparative studies in normal individuals receiving ascorbic acid were carried out, it would appear that conversion of PGA to CF responds to ascorbic acid stimulation in the gouty individual.

The possibility of depletion of the body stores of CF or interference with PGA to CF conversion by medication administered in the course of therapy previous to the time of study is unlikely in view of the lack of effect of the various uricosuric and antigout agents studied. Moreover, some of our patients had received no previous specific gouty therapy.

The absence of change in serum or urine urate levels following the administration of PGA indicates that increasing the tissue stores of PGA and CF does not stimulate uric acid formation or urinary excretion. Thus it would appear that although CF metabolism may be disturbed in patients with gout, it is not the controlling factor in stimulating the formation of uric acid. Similar failure to alter urinary excretion and serum levels of uric acid by the administration of large doses of folic acid both orally and intramuscularly has been reported.[14] Bishop and co-workers[15] have found no significant change in the turnover of uric acid tagged by administration of isotopic glycine in a nontophaceous gouty subject given PGA and vitamin E nor in a tophaceous subject given PGA. Thus our observation that PGA does not stimulate uric acid formation as judged by serum or urine urate levels is in accord with those of others. This, however, does not necessarily exclude the possibility that CF utilization may be accelerated in gouty individuals.

Benedict and associates,[16] using isotopic glycine, have reported a group of gout patients showing evidence of overproduction of urate who also show high urinary urate excretion while receiving low purine diets. In our studies, however, we have been unable to correlate low CF excretion with high uric acid excretion in the three patients with low CF excretion whose urate excretion on low purine diets was studied. This finding appears to be at odds with the hypothesis that a high rate of purine formation and, therefore, increased CF utilization leads to decreased CF excretion. By Benedict's criteria, our patients should have demonstrated high urine urate excretion.

Nichol and co-workers[17] have reported the occurrence of a labile compound related to CF which they have termed CFX. If this is the active form by which transformylation is carried out it is possible that we are not measuring

the true transformylating compound in determining urinary CF. It appears likely, however, that urinary CF is a reflection of the stable form of CFX or its breakdown products.

CF excretion following PGA was sufficiently low in some gout patients in this study as to be comparable to CF excretion in the scorbutic patients reported by Gabuzda and associates.[11] Although a different mechanism for the occurrence of such low urinary figures in the two types of patients may be present, it is worth noting that patients with scurvy are not the only individuals who fail to excrete normal amounts of CF during PGA administration. In other studies we have observed that low urinary CF excretion may also occur in patients with severe liver disease or in myxedema.[18] Such findings may be explained, however, by different mechanisms from that which may be operative in gout.

The question of whether CF is utilized to an abnormal extent or whether it is subject to renal retention in patients with gout will await further work. Studies of the effect of PGA and CF antagonists upon the formation of uric acid may, however, be of value in revealing the underlying defect in patients with hyperuricemia or gout.

SUMMARY

In eight patients with gout and in fourteen normal subjects, urinary excretion of CF and FA was determined during four days of PGA administration orally. In three gouty and three nongouty subjects, urinary excretion of CF was also determined following intramuscular administration of this substance. In selected gouty patients, the effects of ascorbic acid, acetylsalicylic acid, phenylbutazone, colchicine, or probenecid upon urinary CF and FA excretion were also studied.

The average urinary CF in gout patients was significantly less than in normals following either PGA orally or CF intramuscularly. FA excretion following oral PGA was, however, not different in the two groups. CF excretion did not correlate with blood ascorbic acid concentration, although gout patients exhibited lower average concentrations.

Gouty individuals responded normally to ascorbic acid administration with increased urinary CF. Acetylsalicylic acid, phenylbutazone, colchicine, or benemid had no consistent effect upon CF or FA excretion, regardless of the occurrence of increased uricosuria.

PGA administration had no effect upon serum or urine uric acid. Three of the gout patients with very low urine CF excretion did not have excessive urate excretion while receiving low purine diets.

The data suggest that in gout, under the conditions of this study, either increased CF utilization or renal retention of CF is present.

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A COMPARISON OF VOLUMES CALCULATED FROM MEDIAN CIRCULATION TIME AND FROM SLOPE OF HUMAN DYE DILUTION CURVES

HAROLD MILLS,* M.D., AND ALBERT A. KATTUS, JR., M.D.**
LOS ANGELES, CALIF.

INDICATOR dilution curves which have become well accepted as a means for measuring cardiac output[1-5] have also been employed for estimating components of the intrathoracic blood volume. In general two methods have been used in the calculation of these volumes. One employs the formula of Stewart[6] as modified by Hamilton,[4, 7] whereby the cardiac output is multiplied by the mean circulation time, and the other utilizes the slope of the downstroke of the curves as described by Newman.[8] Both methods of calculation have been employed by investigators for the estimation of pulmonary blood volume. The discrepancies noted between the volumes obtained by the different techniques have led us to the present study in which each of 152 single-injection dye dilution curves was analyzed by both methods of calculation and the values compared.†

In 1921, Stewart[6] suggested that the lung blood volume could be calculated by the formula $V = Q \cdot \frac{60}{T}$, where V is the minute output of the heart, Q, the volume of blood in the lungs, and T, the pulmonary circulation time. Since the pulmonary circulation time could not be obtained in the human subject, Hamilton[4, 7] employed the formula $V = MCT \cdot F$, where F is the cardiac output and MCT is the mean circulation time from point of injection to point of sampling (calculated by the formula $\frac{\Sigma\ CT}{\Sigma\ C}$, where C = concentration of dye and T = time in seconds after injection). This method is said to give the volume of blood in the heart, lungs, and great vessels, from the site of injection to the site of sampling. Meier and Zierler[9] in a recently published analysis of this method have affirmed its theoretical validity. Ebert and associates[10] injected dye into the pulmonary artery to determine the volume of blood in the pulmonary vessels, left heart, and larger systemic arteries exclusive of the blood in the right heart and veins. In using Stewart's formula they considered MCT

From the Department of Medicine, University of California Medical Center and the Veterans Administration Center, Los Angeles, Calif.

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*Junior Research Cardiologist, University of California Medical Center, Los Angeles, Calif.

**Associate Professor of Medicine, University of California Medical Center, and Attending Physician, Veterans Administration Center, Los Angeles, Calif.

†In an effort to avoid any controversy over definitions, we have applied "central volume (Stewart)" to the volume measured when Stewart's formula is empolyed and "slope volume" to the volume measured by Newman's formula. The volume measured by Stewart's formula has been termed variously the intrathoracic blood volume, central blood volume, and needle-to-needle volume. The volume measured by Newman's formula has been termed central volume, lung blood volume, and slope volume.

to be the point at which the planimeter divides the area under the dye dilution curve into two equal parts. Hamilton[4] has pointed out that they actually measured the median circulation time and states that this differs from the mean circulation time, especially in asymmetric curves. Dow[11] is quoted as finding this method of calculation to be in error by as much as fifty per cent.

Newman and associates[8] have published a method of calculating a volume within the central circulation from the slope of the downstroke of the dye-dilution curve. This volume is calculated by the formula $V = \frac{F}{S}$ where F' is the cardiac output and S the slope of the downstroke. In their studies the flow through three chambered experimental models built to simulate right heart, lungs, and left heart was measured and dye curves obtained which were of the same form as those obtained from human subjects. It was demonstrated that the volume calculated from the slope of the downstroke was always equivalent to the largest single volume in the system. Subsequent experiments on dogs in which dye was injected directly into the right heart or pulmonary artery and sampling was from the carotid artery or left auricle indicated that the volume which determined the slope in these experiments was located between the pulmonary artery and left auricle. It was postulated that the central circulation behaves as a series of volumes, and the lungs may be considered the largest single volume in the series from which dye is washed out in an exponential fashion. It was suggested that the "slope volume" was in all likelihood a measure of pulmonary blood volume.

METHODS

Dye dilution curves were obtained from normal human subjects and those with heart disease according to the technique of Hamilton, utilizing direct arterial sampling. Subjects were studied in a resting post absorptive state, several on two or more occasions. A No. 17 needle with an attached 8 to 12 inch polyethylene tube (internal diameter 3.5 mm.) was inserted into a procainized brachial artery. After good flow was established, an accurately weighed amount of Evans Blue dye (usually 5 to 7 mg.) was injected as rapidly as possible (in 1 second or less) into a vein in the antecubital fossa of the opposite arm which was elevated on several pillows. Serial samples were collected from the brachial artery with use of test tubes mounted in a disc rotating at a constant speed so that samples were obtained at 1.5- or 2-second intervals. After collection, the samples were centrifuged and serum removed for spectrophotometric determination of dye concentration. The dye concentration curve was then plotted against time on semilogarithmic paper. The straight line downstroke established prior to recirculation of the dye was extrapolated through three cycles of the log paper.

For the sake of simplicity, the median circulation time was used in applying Stewart's formula. Since this method has been criticized by Hamilton,[4] twenty output curves (both symmetrical and markedly asymmetrical) were selected at randon and analyzed by calculating the mean and medium circulation times. These data are shown in Table I. It is seen that almost invariably the mean circulation time is slightly greater than the median circulation time, a factor increasing central blood volume (CVS) by an average of about 8 per cent. This small difference has a negligible effect in the contrast between central volume (Stewart) and slope volume.

TABLE I. A COMPARISON BETWEEN THE MEDIAN AND MEAN CIRCULATION TIMES CALCULATED FROM 20 DYE CURVES SELECTED AT RANDOM FROM SUBJECTS INCLUDED IN TABLES II AND III

SUBJECT	MEDIAN CIRCULATION TIME (SEC.)	MEAN CIRCULATION TIME (SEC.)	DIFFERENCE (SEC.)	CENTRAL VOLUME (STEWART) USING MEDIAN CIRCULATION TIME (LITERS)	CENTRAL VOLUME (STEWART) USING MEAN CIRCULATION TIME (LITERS)	DIFFERENCE (LITERS)
1	60.5	64.3	+3.8	3.88	4.12	+0.24
2	44.0	48.3	+4.3	1.49	1.63	+0.14
3	65.0	73.2	+8.2	3.88	4.37	+0.49
4	40.5	44.8	+4.3	4.53	5.01	+0.48
5	52.0	59.2	+7.2	4.16	4.74	+0.58
6	22.5	24.1	+1.6	2.85	3.03	+0.18
7	24.0	26.4	+2.4	3.50	3.85	+0.35
8	20.5	22.1	+1.6	2.79	3.01	+0.22
9	20.0	22.3	+2.3	3.13	3.49	+0.36
10	20.0	23.9	+3.9	1.58	1.89	+0.31
11	19.5	22.8	+3.1	1.86	2.18	+0.32
12	69.0	79.0	+10.0	3.10	3.56	+0.46
13	23.0	23.1	+0.1	2.26	2.27	+0.01
14	39.0	41.7	+2.7	2.39	2.56	+0.17
15	31.0	31.4	+0.4	2.68	2.71	+0.03
16	43.0	43.9	+0.9	7.72	7.88	+0.16
17	29.3	33.3	+4.3	3.18	3.72	+0.54
18	31.0	33.1	+2.3	3.04	3.25	+0.21
19	25.5	37.4	+1.9	4.20	4.43	+0.23
20	20.0	19.7	−0.3	2.71	2.68	−0.03
Average	35.57	38.7	3.23	3.25	3.52	0.28

RESULTS

The data are listed in the accompanying tables and illustrated in Fig. 1. The median circulation time is the point at which the dye dilution curve is divided into two equal parts. Central blood volume (Stewart) was calculated by Stewart's formula utilizing the median circulation time. Slope volume (SlV) was calculated by Newman's method. The difference between the two volumes was obtained by subtracting SlV from CVS, for in all instances CVS was larger. CVS and SlV are also expressed as per cent of total blood volume. No attempt is made to separate patients according to specific disease as this will be done in subsequent articles.

Table II contains the data obtained in resting normal subjects and Table III contains data from subjects with known or suspected cardiac disease. In

TABLE II. SUMMARY OF DATA OBTAINED FROM 13 CARDIAC OUTPUT STUDIES IN NORMAL RESTING SUBJECTS

	MEAN AND STANDARD DEVIATION
Median circulation time	30.1 ± 7.69 seconds
Central volume (Stewart)	1.76 ± 0.42 liters per M^2 body surface area
Slope volume	0.53 ± 0.20 liters per M^2 body surface area
Difference (CVS—SV)	1.23 ± 0.35 liters
Per cent difference	69.58 ± 8.18
$\frac{\text{Central volume (Stewart) } 0.100}{\text{Blood volume}}$	60.55 ± 11.32 per cent
$\frac{\text{Slope volume } 0.100}{\text{Blood volume}}$	18.50 ± 5.83 per cent

TABLE III. SUMMARY OF DATA OBTAINED IN 139 CARDIAC OUTPUT STUDIES IN RESTING SUBJECTS WITH CARDIAC DISEASE OR SUSPECTED OF HAVING CARDIAC DISEASE

	MEAN AND STANDARD DEVIATION
Median circulation time	34.4 ± 11.74 seconds
Central volume (Stewart)	1.51 ± 0.45 liters per M^2 body surface area
Slope volume	0.56 ±0.28 liters per M^2 body surface area
Difference (CVS—SV)	0.95 ± 0.32 liters
Per cent difference	62.9 ± 13.68
$\frac{\text{Central volume (Stewart) } 0.100}{\text{Blood volume}}$	49.3 ± 11.44 per cent
$\frac{\text{Slope volume } 0.100}{\text{Blood volume}}$	17.7 ± 9.37 per cent

all instances SlV is considerably smaller than CVS, a fact also demonstrated in Fig. 1 where SlV is plotted against CVS. In normal subjects CVS comprises approximately 50 to 70 per cent of the total blood volume, while SlV ranges from 12 to 25 per cent of total blood volume and SlV averages 30 per cent of the CVS. In the subjects with known or suspected cardiac disease, the average values were not strikingly different from the normals, except for the fact that the SlV in this group averages 37 per cent of the CVS.

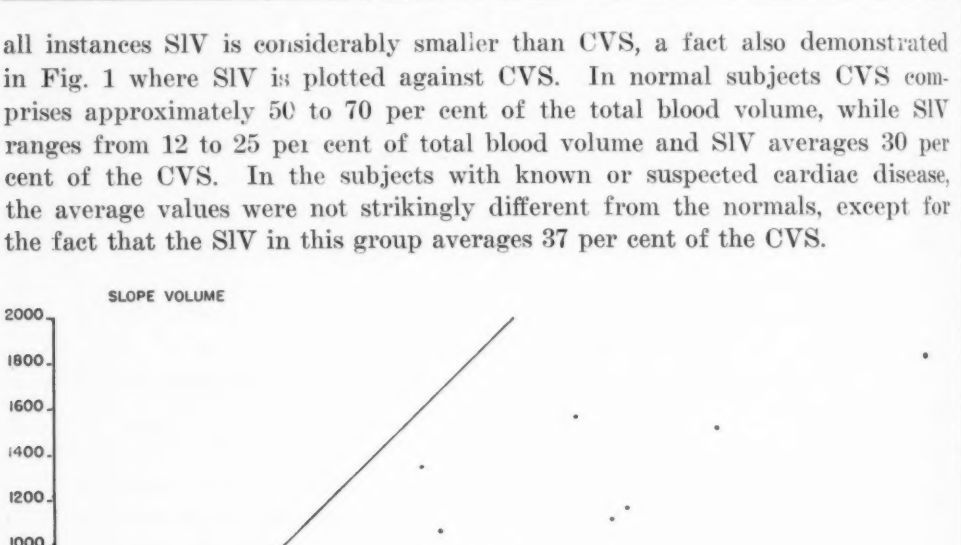

Fig. 1.—Simultaneously obtained slope volumes and central volume (Stewart) are plotted against each other. It is seen that the line of regression is markedly smaller than 45 degrees and that slope volume is invariably less than central volume (Stewart). The ⊗ represents normal subjects, and the ● represents subjects with known or suspected cardiac disease.

DISCUSSION

The data reveal that the slope volume is invariably smaller than CVS. The difference of approximately 1 to 2 liters between the two may be explained by consideration of the anatomical limits of these volumes. With use of the formula $V = \frac{F}{S}$, the wash-out slope of the dye dilution curve is deter-

mined by the largest single volume in series. Thus, if the antecubital fossa is the site of indicator injection, it is likely that the largest volume in the system is the lung blood volume and that this volume determines the washout slope. In our experiments SlV averaged 17 to 18 per cent of total blood volume, figures which correlate well with other published data for estimating pulmonary blood volume.[13-15]

Our data reveal that CVS comprised 50 to 70 per cent of total blood volume. With the site of injection at the antecubital fossa, the time required for the dye to reach the heart will increase the calculated value by raising the MCT. Blumgart and Weiss[14] found the circulation time from antecubital fossa to right auricle to be 6.7 seconds. The shorter time required for dye to travel from the left ventricle to the site of sampling will also contribute to the MCT. Thus the volume measured must include blood not only within the vein carrying dye to the heart but also all venous blood which arrives at the right heart simultaneously with the dye and mixes with it. Furthermore the volume must include blood in all arteries having comparable simultaneous dye concentrations as that at the sampling point. It would appear, therefore, that CVS must include not only intrathoracic blood but also venous and arterial blood which is contained within vessels below the diaphragm as well as in the head and neck. This point has also been commented on by Coe, Best, and Lawson.[16]

Other workers who have compared the slope volume with the central volume (Stewart) have found ratios of $\frac{\text{SlV}}{\text{CVS}}$ to vary from 0.21 to 0.66.[17-20] Our ratios of 0.30 for normals and 0.37 for cardiac patients compare favorably with those of Dow,[17] Shadel and associates,[18] and Tompkins and associates,[19] all of whom found ratios of 0.21 to 0.31 in dogs and human subjects. Rabinowitz and Rapaport[20] found a much higher ratio of 0.66. Perusal of the tables in the article of Hamilton and associates[7] reveals the "intrathoracic" blood volume in human subjects to range around 1,500 ml. per square meter (range approximately 1,000 to 2,150 ml. per square meter). The site of injection was the antecubital fossa, the site of sampling, the femoral artery. These figures are similar to the CVS obtained in our experiments. Furthermore, Ebert and associates,[10] using Stewart's formula for calculating the volume of blood from pulmonary artery to femoral artery, found much smaller volumes (mean—1,160 ml.) Doyle and co-workers[5] also found much smaller volumes (mean—634 ml. per square meter) in normal subjects using the pulmonary artery as the site of dye injection and Stewart's formula for calculation. Ebert's group calculated the median circulation time following injection of dye into the pulmonary artery and determined the mean to be 10.2 seconds. The mean value determined by Chapman and Fraser[21] injecting dye through a catheter threaded 30 cm. toward the heart through a vein in the antecubital fossa was 18.2 seconds. Our mean was 30.1 seconds in normal resting subjects. Thus the further the site of indicator injection is from the heart, the greater the MCT and the greater the volume calculated by Stewart's formula.

It is clear that if the antecubital vein is used for indicator injection, calculation of the slope volume gives values much smaller than the central volume of

Stewart. The extent to which slope volume is modified by injection site remains a point of controversy. Hetzel and co-workers[22] compared "central blood volume (Stewart)" and "lung blood volume (Newman)" after injecting dye into the pulmonary artery, superior vena cava, and peripheral vein. The central volumes (Stewart) obtained from pulmonary artery dye injections were considerably smaller than those calculated after peripheral vein injection, the mean difference being 0.75 l per square meter. Similarly slope volumes were smaller with central dye injections than they were with peripheral dye injections, the mean difference being 0.21 l per square meter. A possible objection to these data lies in the fact that peripheral injections were made through a catheter. With the catheter in the vein, the distal segment would presumably be blocked off so that dye could not be washed into the heart by the normal flow of blood. This would tend to prevent rapid delivery of dye to the right heart and thus raise slope volume. It is possible that the SlV calculated after peripheral injection may include blood in the ventricles, atria, and aorta,[23] although data supporting this contention have not been presented. However, injection of dye directly into the pulmonary artery may yield falsely low values, for, as suggested by Conn,[24] this site of injection may predispose to incomplete mixing and to laminar flow of dye preventing uniform distribution of dye to the lung blood vessels. However, parallel mixing within the left auricle would at least in part prevent error from this source. The significance, if any, of differential red cell and plasma velocity, of injection rate, and of laminar flow within the great vessel streams entering and leaving the heart remain to be determined. Finally, the amount of blood withdrawn for sampling may affect SV and CVS. Lawson and associates[25] have demonstrated in dogs that blood lost through sampling may increase the washout slope as well as decrease cardiac output.

It is evident from both theoretic and experimental evidence that SlV is invariably smaller than CVS. Furthermore, there is a wide range of differences between SlV and CVS which makes it impossible to derive SlV from CVS by subtraction of a constant amount. The evidence therefore suggests that slope volume constitutes a more reliable index of pulmonary blood volume than CVS. Delineation of the exact anatomical limits of the slope volume must await further study.

SUMMARY

1. A comparison is made between the Newman and Stewart methods for calculating central blood volume or its components from indicator dilution curves obtained from 13 normal resting subjects and 139 resting patients with known or suspected cardiac disease.

2. With use of the antecubital fossa as the site of indicator injection, the slope volume derived from the formula of Newman comprises approximately 18 per cent of total blood volume in both the normal and cardiac groups. The volume derived from Stewart's formula averages 60 per cent of total blood volume in normals and 50 per cent in the cardiac group.

3. The slope volume appears to be a more definitive volume and probably a more reliable index of lung blood volume than the volume calculated utilizing Stewart's formula.

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EFFECT OF SPHEROCYTOSIS ON VOLUME OF TRAPPED PLASMA IN RED CELL COLUMN OF CAPILLARY AND WINTROBE HEMATOCRITS

FRANK W. FURTH, M.D.*
ROCHESTER, N. Y.

ACCURATE estimation of the amount of plasma trapped amongst the packed red cells in hematocrit tubes after centrifugation is necessary for proper computation of packed red cell volume and of the red cell dimensions. An estimate of trapped plasma is also needed in determinations of blood volume depending upon accurate measurement of packed red cell column. The problem of trapped plasma and correction of the packed cell volume in hematocrit tubes has recently been studied by a number of investigators, who have employed several different techniques.[1-3]

The reported estimates of trapped plasma are somewhat variable. The variability has been attributed to differences in technique of measurement and to differences in amount of centrifugal force and time of application of the force.[4, 5] Increased centrifugal force and/or increased time of exposure to the force has been shown to decrease the amount of intercellular plasma.[4, 5] However, it has been demonstrated that with the conditions of centrifugation usually employed with Wintrobe hematocrit tubes, the variation from individual to individual may be significant.[1, 4] Little attention has been paid to those factors intrinsic to the red cell which can influence the amount of intercellular plasma in the red cell column of the hematocrit. These factors include the size and shape of the erythrocyte and its deformability. A few observations have been reported on the effect of abnormal cell shapes and sizes on plasma trapping. Chaplin and Mollison[4] concluded that erythrocyte size influenced the amount of intercellular plasma, and that the shape of the red cell caused an insignificant alteration in trapped plasma. However, the intercellular plasma was determined in blood from only one individual with hereditary spherocytosis.

This study was initiated to determine the effect of an abnormality in the shape of the erythrocytes (spherocytosis) on the amount of intercellular plasma in the red cell column of the centrifuged capillary and Wintrobe hematocrit tubes. The amount of trapped plasma (actually serum) was determined by a direct method employing Cr^{51}-tagged serum proteins. Determinations were made on blood obtained from normal individuals and from patients with hereditary

From the Department of Medicine, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

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*Special Fellow, National Heart Institute, U.S. Public Health Service. Present address: Department of Medicine, University Hospital of the Good Shepherd, Syracuse, N. Y.

spherocytosis (hereafter abbreviated HS). The changes in shape and in volume of both normal and HS red cells on sterile incubation at body temperature have been studied recently in several laboratories.[6-8] These changes are thought to have an important bearing on the pathogenesis of anemia in hereditary spherocytosis since HS cells trapped in the spleen, or incubated in vitro, undergo sphering more rapidly than do normal red cells. In this study, measurements were made on normal and HS blood after incubation for periods of 24 and 48 hours.

METHODS

Collection and Preparation of Blood.—Fifteen milliliters of blood was obtained from the antecubital vein by aseptic technique and immediately transferred to a sterile 125 ml. flask containing 18 glass beads of approximately 4 mm. diameter. The closed flask was agitated until the blood was defibrinated. With use of sterile pipettes, 4 to 5 ml. aliquots of blood was transferred to each of three sterile capped tubes. One of these tubes was used immediately for the estimation of trapped intercellular serum. The other two tubes were placed in an incubator at a temperature of 37° C. At intervals of 24 hours and 48 hours a tube was removed and another estimation of trapped serum was made. Samples of blood were obtained from 12 normal adults and from 10 adults with hereditary spherocytosis. Seven of the individuals with hereditary spherocytosis had been splenectomized. Since no anticoagulant was added to the blood and the fibrinogen was removed, the liquid portion of the blood used in this study was serum rather than plasma. Defibrinated blood was used throughout this investigation because all studies in this laboratory on autohemolysis and on changes in osmotic and mechanical fragility of incubated red cells had been made with defibrinated blood rather than with blood containing anticoagulant.

Cr^{51} Labeling of Serum.—Each 5 ml. aliquot of blood was centrifuged for 3 to 5 minutes at 3,000 r.p.m., and the supernatant serum was removed and transferred to a test tube containing 40 to 50 μc of Cr^{51} as chromic chloride. The chromic chloride was dissolved in 0.05 to 0.15 ml. of distilled water and represented 0.03 to 0.15 mg. of chromium. After thorough mixing the serum was allowed to stand at room temperature for 5 to 10 minutes to remove flecks of fibrin. The serum was next recombined with the red cells and remaining buffy coat from which it had been separated and the cells were resuspended by inversion. After thorough mixing the hematocrit tubes were filled.

Wintrobe Hematocrit Method.—The Wintrobe tubes used in this study had an internal diameter of 3.0 mm. and a calibrated column length of 10 cm. Three tubes were filled to the "10" mark from each aliquot of blood. A centrifuge* with a radius of 20 cm. to the bottom of the cup (15 cm. to the middle of the hematocrit tube) was used for centrifugation. The tubes were centrifuged at 3,000 r.p.m. for 30 minutes (relative centrifugal force = 1,500 × g), after which the level of the packed red cell column was recorded. A 0.02 ml. aliquot of the supernatant serum was removed with a hemoglobinometer pipette from each tube for radioactivity determination. With use of a capillary pipette the remainder of the serum was removed with care not to disturb the red cell column. In order that the upper portion of the tube might be rinsed, saline solution was added cautiously and then removed with gentle suction. This was repeated 4 times. After the fourth washing with saline the packed red cells were transferred quantitatively to a vial for radioactivity determination. The transfer was made with a Pasteur pipette, with use of small amounts of distilled water until the final volume in the counting vial was 3 ml.

Capillary Hematocrits.—Glass capillary tubes† with an internal diameter of 0.5 to 1.5 mm. and a length of 75 mm. were used. One end of the capillary tube was inserted in a well-mixed sample of blood labeled with Cr^{51} as outlined above. The tube filled by

*Model V, Size 2 centrifuge and a capillary hematocrit centrifuge, both manufactured by International Equipment Co., Boston, Mass., were used in this study.

†Capillary hematocrit tubes were obtained from A. S. Aloe Company, St. Louis, Mo.

capillarity to a total blood column length of 55 to 65 mm. The "empty" end of the tube was then quickly sealed with a gas flame. The tubes were centrifuged* for 4 minutes at 10,000 r.p.m., with a radius of 9 cm. to the bottom of the tube. (RCF = approximately 10,000 × g). After centrifugation, the total length of the blood column, the length of the packed cell column and plasma column were recorded in millimeters. By means of a small high-speed abrasive disc the capillary tube was cut 1 to 2 mm. below the plasma-packed cell juncture. The length of tubing containing almost all of the red cell column was measured, another cut was made close to the bottom of the tube, and both pieces of this tube were placed in a vial containing 3 ml. of distilled water for radioactivity determination. By means of vigorous shaking, the red cells were removed from inside the capillary tube, thereby making a uniform solution of hemoglobin in the vial. A portion of the capillary tube 20 to 25 mm. in length containing plasma was cut just above the serum-packed cell interface and the length was recorded. This portion of the capillary tube was then placed in a vial containing 3 ml. of distilled water for radioactivity determination. By means of vigorous shaking, the serum was distributed in the water in the vial. The radioactivity in the red cell column and serum was then determined and expressed in number of counts per minute per millimeter of length of capillary tube. Since diameters amongst the capillary tubes varied, the specific activities of packed cell column and serum of the same tube were paired in calculating the serum trapping in each separate tube.

Correction for Cr^{51} Absorbed by Red Cells.—Although it has been reported that the erythrocyte does not absorb appreciable amounts of chromic ion in the presence of serum and that the chromic ion firmly attaches to serum protein,[9] it was found that a small but unpredictable amount of activity (not more than 10 per cent of the added $Cr^{51}Cl_3$) became attached to the red cells. It was necessary to correct the specific activity of the packed cell column for the activity attached to the cells. In all instances, after the capillary and Wintrobe tubes had been filled, the remaining blood containing Cr^{51}-labeled serum was centrifuged and the serum removed. Twenty volumes of an 0.85 per cent solution of sodium chloride was added, the cells suspended, and centrifugation repeated. The cells were washed in this manner three times. Following the last wash, the cells were suspended in a volume of saline sufficient to approximate the hematocrit of the original blood. Wintrobe and capillary hematocrit tubes were filled with this suspension. The specific activity of the packed cells and supernatant saline was determined by the method previously described.

Radioactivity Determinations.—All radioactivity measurements were made with a well-type scintillation counter containing a Harshaw thallium-activated sodium iodide crystal. All counts were corrected for natural background and were expressed as counts per minute. Sufficient total counting time was allowed to reduce the random count error below 2 per cent. All samples for counting were made up to a total volume of 3 ml. in 15 × 60 mm. glass vials.

Calculations—

Wintrobe tube: The specific activities per cubic millimeter of the packed red cell column and of the serum were calculated. The activity of the cell column was corrected for activity absorbed on the red cells. By dividing the specific activity per cubic millimeter of serum into the corrected activity per cubic millimeter of red cell column, the amount of trapped serum was determined.

Volume of packed cell column = (Vol_{pcc})

Radius of the tube = 1.5 mm. 1 per cent mark on hematocrit tube = 1.0 mm.

$Vol_{pcc} = \pi\ r^2h = 7.07 \times$ length of packed cell column in millimeters

Specific activity of packed cell column (SA_{pcc})

$$\frac{\text{CPM in total packed cell column}}{Vol_{pcc}} = SA_{pcc}$$

Corrected Specific Activity of Packed Cell Column.—Specific activity of washed red cells was determined in the same manner as for the determination of specific activity of

*A Model V, Size 2 centrifuge and a capillary hematocrit centrifuge, International Equipment Company, Boston, Mass.

the packed cell column (SA_{pcc}). The CPM per cubic millimeter of washed red cells was then subtracted from the CPM per cubic millimeter of packed cell column to give a corrected SA_{pcc}.

Specific activity of serum (SA_s):

Aliquot of serum used for radioactivity determination = 20 cubic millimeters

$$\frac{\text{CPM in serum aliquot}}{20} = \text{CPM per cubic millimeter serum } (SA_s)$$

Per cent of packed cell column that is serum

$$\frac{\text{Corrected } SA_{pcc}}{SA_s} \times 100 = \text{per cent trapped serum}$$

Capillary Tube.—The specific activity of packed cell column and serum were determined and expressed as CPM per millimeter length of capillary tube. The specific activity of the packed cell column was corrected for Cr^{51} activity within the red cell by subtracting the mean specific activity of the packed cell column of three capillary tubes of washed red cells from the specific activity of the packed cell column of unwashed cells. By division of the specific activity of the packed cell column, the portion of the packed cell column that is serum was calculated and expressed in per cent. The calculations were identical to those used for the Wintrobe method.

RESULTS

The method employed in this study determines directly the amount of serum trapped among the red cells in the hematocrit tube. The values obtained are calculated to express the per cent of the observed cell column which consists of serum. Therefore, in order to obtain the corrected or absolute packed cell volume the observed reading is multiplied by the per cent correction and the result subtracted from the observed reading.

In the construction of the graphs in Figs. 1 and 2, each reading of packed red cell volume was corrected as described and this value entered on the appropriate graph. The observed packed cell volume is plotted on the abscissa, and the corrected value on the ordinate. The solid line on each graph represents the relationship between abscissa and ordinate if no correction is applied. The dotted line represents an arbitrary "best fit" line for the observations of the corrected cell volumes.

Table I shows the mean per cent correction for trapped serum for normal blood and blood obtained from individuals with hereditary spherocytosis, with no incubation and following 24 and 48 hours of sterile incubation, utilizing the

TABLE I. SUMMARY OF DATA ON CORRECTION OF PACKED RED CELL VOLUMES

	CAPILLARY TUBES			WINTROBE TUBES		
	INCUBATION			INCUBATION		
	NONE	24 HRS.	48 HRS.	NONE	24 HRS.	48 HRS.
No. normal donors	10	16	12	7	10	11
No. observations	36	53	40	21	30	33
Packed cell volume, observed range per cent	38-54	14-56	14-55	39-53	14-56	14-59
Mean per cent correction	2.0	2.2	4.8	3.6	3.9	10.6
Standard error	0.30	0.16	0.64	0.40	0.20	0.60
No. HS patients	12	14	13	12	11	12
No. observations	38	43	40	36	33	36
Packed cell volume, observed range per cent	28-53	18-57	14-56	18-53	18-58	14-61
Mean per cent correction	4.9	3.5	8.1	6.6	6.7	11.6
Standard error	0.75	0.29	0.81	0.76	0.70	0.76

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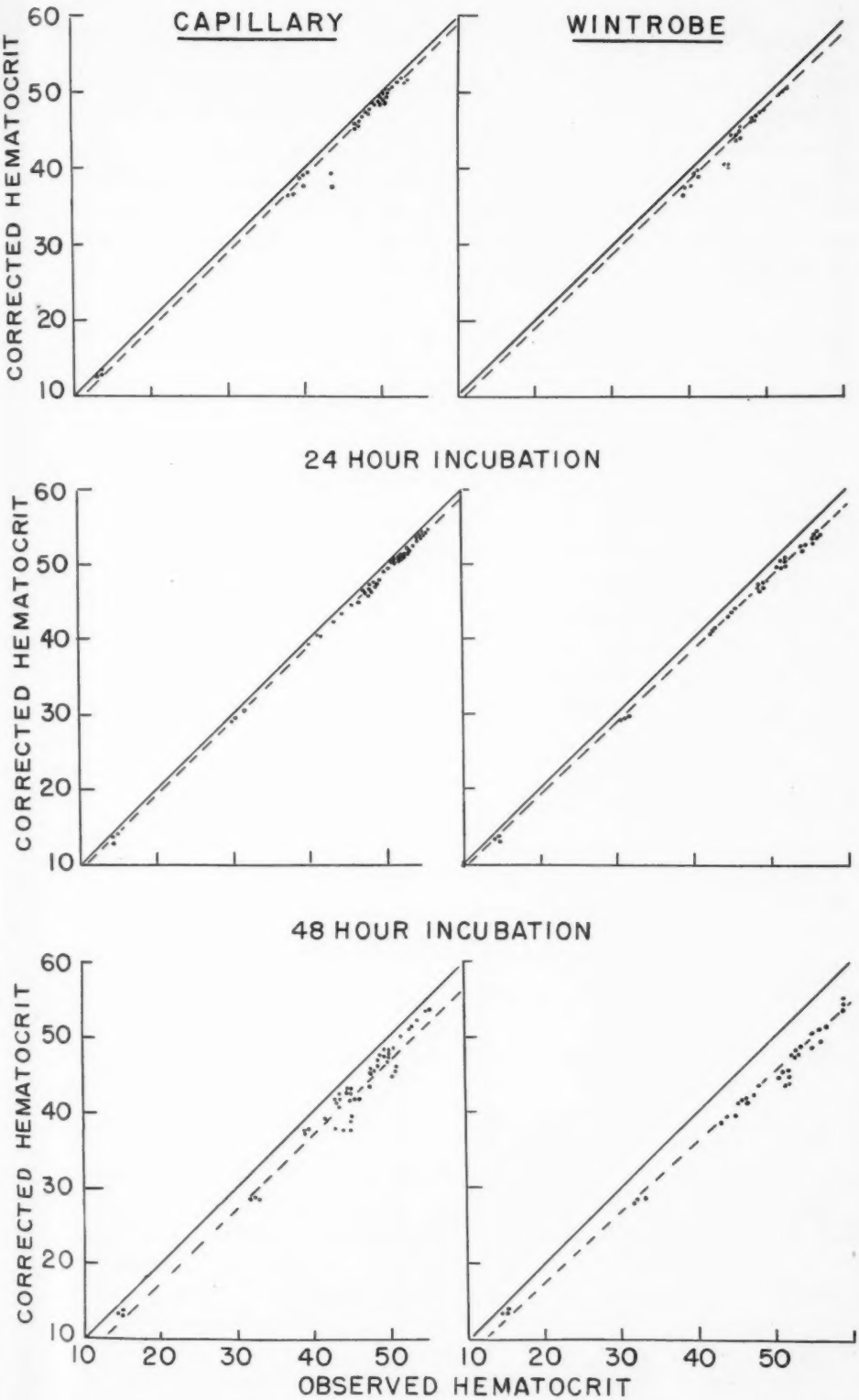

Fig. 1.—Fresh normal blood, no incubation.

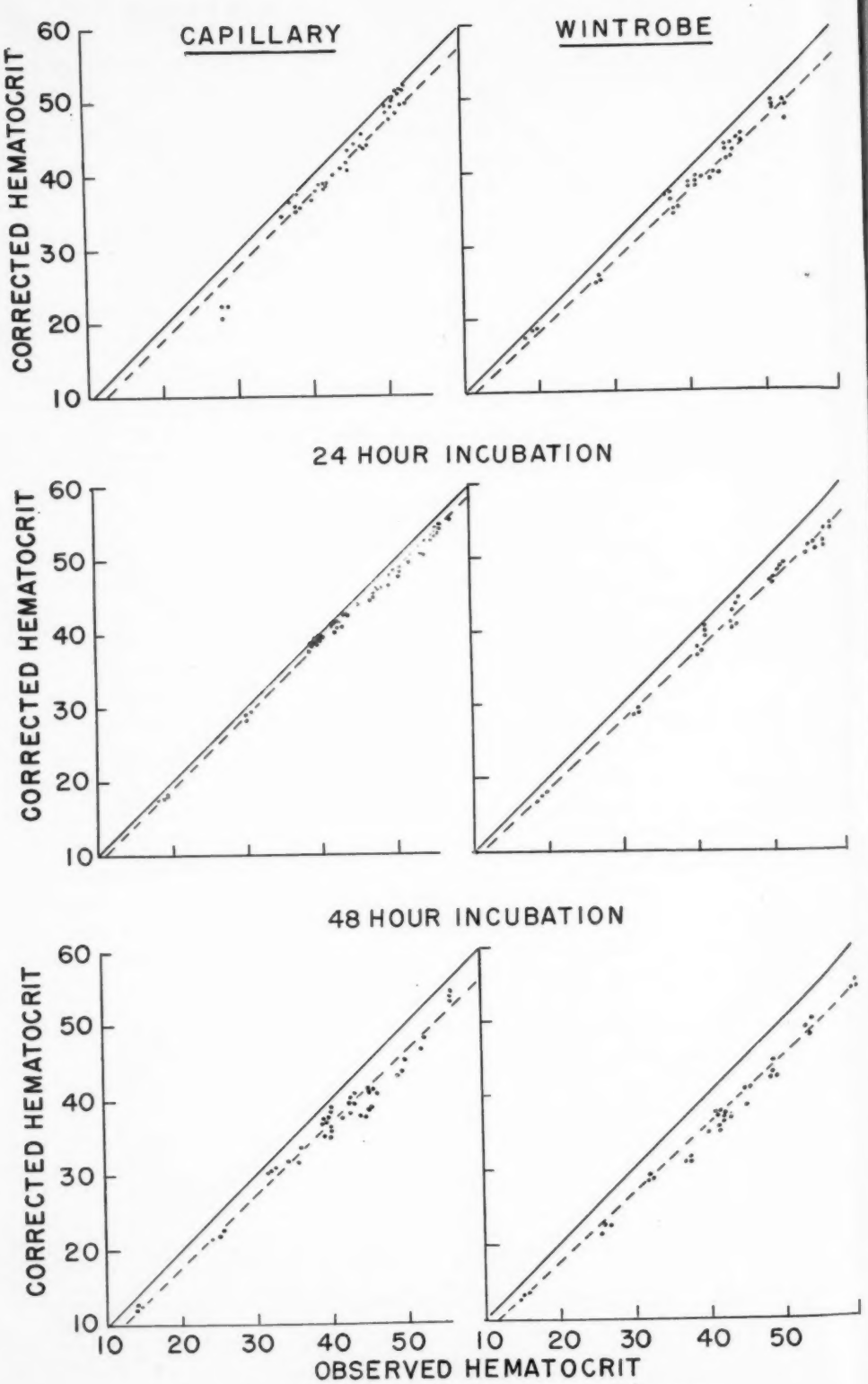

Fig. 2.—Fresh hereditary spherocytosis blood, no incubation.

Wintrobe and capillary hematocrit methods. The values in this table were calculated by determining the mean of 3 or 4 observations on each subjects's blood under the conditions given above. The mean of all the values so obtained was determined and from this the standard error calculated. Values were obtained on two normal subjects on three occasions, and on two HS individuals on two and three occasions, respectively. In the calculation of the group mean, the values obtained from these individuals on separate occasions were considered as separate subjects.

Previous observers have found a positive correlation between the per cent of trapped plasma and the observed hematocrit, which indicated that the per cent of trapped plasma increases in a direct manner with the observed hematocrit.[4, 10] No such correlation was found in this study in which mean per cent trapped plasma was determined on undiluted samples of blood obtained from randomly selected normal subjects, and from patients with hereditary spherocytosis. Since no correlation could be established, the mean per cent correction and standard error for intercellular serum were calculated over the range of observed packed cell volumes shown in the table. Although the relative amount of trapped serum (as expressed by the percentage correction) shows no correlation with packed cell volume, it is obvious that the absolute amount of intercellular serum varies directly with the height of the packed red cell column.

An examination of the results reported in Table I and on the graphs reveals the following:

1. The amount of intercellular serum in the cell column of the capillary hematocrit tube is consistently less than the amount of intercellular serum in the Wintrobe tube.

2. With both the capillary and Wintrobe methods the mean per cent correction for trapped serum is greater for blood obtained from individuals with hereditary spherocytosis than for normal blood.

3. Sterile incubation of blood from both normal donors and from patients with hereditary spherocytosis increases the amount of intercellular plasma with both hematocrit methods.

4. The variation in per cent of intercellular serum is greater with HS blood than with normal blood under identical conditions of centrifugation.

DISCUSSION

No attempt was made in this study to consider the effect of the physical factors which cause variations in the amount of intercellular serum within the packed red cell column of the hematocrit tube. Previous studies have shown that the amount of intercellular plasma varies in an inverse manner with force applied to the cell column and the duration of the force application.[1, 3-5] It has also been demonstrated that the intercellular plasma is not uniformly distributed throughout the cell column; i.e., the proportion of intercellular plasma decreases toward the bottom of the cell column.[3, 5] The speed and duration of centrifugation used in this study were chosen because these conditions appear

to be those most commonly used in clinical laboratories. These conditions are not considered necessarily optimal in respect to reducing intercellular plasma to minimal values.

The amount of intercellular plasma appears to be a reflection of the efficiency of red cell packing. If all intercellular space were obliterated there would be no "trapped plasma" and the packing would be optimal. The degree of packing represents an interplay between the physical forces applied to the suspended red cells and the intrinsic properties of the red cell. Studies of the effect of varying the physical force applied have shown that packing can be increased by increased force and by prolonging the exposure to a given force.[4] In this study the external physical forces were kept constant. The intrinsic red cell properties modifying the packing include the shape of the cell, the size of the cell, and the "compliance" or distortability of the cell. The differences in intercellular serum observed during this study would appear related to these properties of the red cell.

The red cells of individuals with hereditary spherocytosis have an increased thickness-diameter ratio. Sterile incubation at 37° C. of both normal and HS blood causes an increase in thickness-diameter ratio of the red cells, increased susceptibility to mechanical destruction and to lysis in hypotonic solutions of sodium chloride.[6, 11] These changes on incubation occur more rapidly with HS cells than with normal erythrocytes. The packing of suspended discs would be expected to be greater than that of spheres if the surface area of both were the same. It could be predicted, therefore, that on the basis of increased sphering, the intercellular plasma would be greater in HS blood and that the amount would be further increased in incubated blood. The results of this study support this thesis.

The mean corpuscular volume in hereditary spherocytosis is usually normal[12] in the absence of reticulocytosis (as after splenectomy). However, incubation of either normal or HS blood for 24 hours significantly increases the mean erythrocyte volume. During the second 24-hour period of incubation, both normal and HS cells shrink so that the volume approximates that recorded prior to incubation.[7, 13] It is difficult to assess the role of these volume changes on the intercellular plasma of incubated blood.

It can be assumed that with a sufficient force applied for an optimal time, red cell distortion could occur to the extent that little if any intercellular plasma would remain. In this study, small but appreciable amounts of intercellular serum remained after the application of 10,000 g for 4 minutes with normal cells. Increasing amounts of intercellular serum were found in normal blood incubated for 24 and 48 hours. In addition to the volume and shape changes, the normal cells may become less plastic following incubation and less easily distorted. This may also be true for HS erythrocytes on incubation. The force-time relation to which the erythrocytes were subjected in this study was apparently incapable of distorting the cells to an extent which would eliminate the intercellular serum. However, the difference between the trapped serum in the capillary tube and in the Wintrobe tube suggests that increased force will cause increased cell distortion.

Aside from the implications concerning intrinsic red cell properties, the results of this study make available correction factors which are useful in the calculation of erythrocyte volume in normal and HS blood before and after incubation. The range of values obtained is in general agreement with those previously obtained for normal blood in the Wintrobe tube.[1-5] The small quantity of intercellular serum in the erythrocyte portion of the capillary hematocrit suggests that this method provides for more efficient red cell packing and closely approximates the absolute red cell volume. The decreased plasma trapping, short centrifugation time, and small volume of blood required for the capillary method are noteworthy advantages of this method.

SUMMARY

1. With use of Cr^{51}-labeled serum, measurements were made of the amount of serum trapped in the red cell column of Wintrobe and capillary hematocrit tubes containing defibrinated blood from normal donors and from patients with hereditary spherocytosis. The influence of incubation for 24 and 48 hours on the trapped serum was also determined.

2. The amount of trapped serum was less in the capillary hematocrit tube than in the Wintrobe hematocrit tube, but greater with blood from patients with hereditary spherocytosis than with normal blood in both tubes. Incubation of both types of blood increased the amount of intercellular serum in the red cell column of the hematocrit tubes.

3. The differences in amount of trapped serum determined in this study appear to be related principally to differences in erythrocyte shape and distortability rather than in cell volume.

4. Figures are given for correction of observed hematocrit readings for trapped serum in normal blood and in blood from persons with hereditary spherocytosis.

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ENDOCRINE INFLUENCES UPON THE UPTAKE OF COLLOIDAL THORIUM BY THE RETICULOENDOTHELIAL SYSTEM

SHERWOOD M. REICHARD, PH.D.,* ABRAHAM EDELMANN, PH.D.,** AND ALBERT S. GORDON, PH.D.
NEW YORK, N. Y.

INTRODUCTION

IT HAS been demonstrated[1-3] that adrenalectomy in the rat results in a diminution in the concentration and activity of the splenic macrophages. The administration of large quantities of whole adrenal cortical extract was effective in preventing these effects. These studies employed chemical analyses for the determination of thorium within the tissues. Since thorium is a naturally occurring radioactive element, its presence within the phagocytic cells of the reticuloendothelial organs can also be quantitated on the basis of its radiochemical characteristics. In the present study both chemical and radiochemical procedures were utilized in examining the actions of some hormonal factors, administered singly and in combination, upon the uptake of colloidal thorium dioxide by the splenic and hepatic tissues of adrenalectomized and hypophysectomized rats.

MATERIALS AND METHODS

Young adult male albino (Wistar) and female hooded (Long-Evans) rats, with initial body weights ranging between 135 to 160 grams, were used in these studies. They were maintained on a well-balanced diet with access to drinking water at all times and were kept in rooms which were thermostatically controlled at 75° ± 1° F. Bilateral adrenalectomies were performed by the usual dorsal approach and hypophysectomies by the parapharyngeal approach. All animals were checked at autopsy to determine completeness of the operations; any found to be incomplete were discarded and not included in the data. The drinking fluid of adrenalectomized rats consisted of 1 per cent NaCl, whereas the hypophysectomized rats had access to 5 per cent glucose. In addition, the diets of hypophysectomized rats were fortified with bread, milk, fresh meat, beef suet, fresh oranges, and raw carrots.

Cortisone (Compound E of Kendall) was used as a saline suspension, and hydrocortisone (Compound F of Kendall) as an oil suspension. Growth hormone (GH; Raben-Westermeyer preparation) was triturated, suspended in distilled water, and adjusted to pH 3. Adrenocorticotrophic hormone (ACTH; 20 units per milligram) was triturated and suspended in sesame oil containing 2 per cent aluminum monostearate.

Thorotrast is a stabilized colloidal suspension of thorium dioxide, containing 24 to 26 per cent ThO_2 by volume. A modification of the method of Harrington and Huggins[4]

From the Departments of Biology, Graduate School of Arts and Science, New York University, New York, N. Y., and Brookhaven National Laboratory, Upton, N. Y.

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*Present address: Armed Forces Institute of Pathology, Washington 25, D. C.
**Present address: Nuclear Science and Engineering Corporation, Pittsburgh, Pa.

and of Gaunt and Wright[5] was used to determine chemically the quantities of thorium in the tissues. The entire spleen and approximately three-fourths of the liver of each animal were cut into small pieces, placed in beakers, and dried in a hot-air oven at 95 to 100° C. for 24 hours. Digestion was accomplished by allowing the material to stand several days in a mixture of 1 ml. of perchloric acid, 1 ml. of nitric acid, and 4 ml. of sulfuric acid. The tissues were heated carefully until complete solution of ash occurred. To ensure complete solution in the liver samples where a greater bulk was present, it was necessary to use additional acid. These tissues were transferred into Erlenmeyer flasks to which 20 ml. of sulfuric acid, 8 ml. of perchloric acid, and 6 ml. of nitric acid were added, a little less than the quantities used by Kennedy.[6] After these materials had cooled, 25 Gm. of pulverized ice, prepared from distilled water, was added to each to form the octahydrate of the thorium sulfate. Concentrated NH_4OH was added until a pH of approximately 8 was attained, and the contents were transferred to 50 ml. centrifuge tubes. These were allowed to stand overnight. The precipitate of thorium hydroxide was centrifuged, washed once with distilled water, centrifuged again, and the supernatant fluid discarded. The precipitate was dissolved in 10 to 15 drops of concentrated HCl. To each tube was added 3 ml. of 10 per cent oxalic acid solution, and these were allowed to stand overnight. The thorium oxalate precipitate was washed three times with distilled water, recentrifuged each time, and dissolved in 4 ml. of 25 per cent sulfuric acid. The contents of the tubes were then titrated with 0.01 N $KMNO_4$ at 90° C. Thorium dioxide in milligrams was calculated by multiplying the volume of $KMNO_4$ in milliliters by the factor 0.66 (1 ml. of 0.01 N $KMNO_4$ equals 0.66 mg. ThO_2). For valid comparisons between different experiments, the results were expressed as milligrams ThO_2 per gram dried tissue.

For radiochemical analyses, the entire spleens and halves of livers for duplicate samples were cut into small pieces and spread evenly on the bottom of previously weighed 20 by 85 mm. screw-cap glass vials. The gamma radiation of each sample was then measured in a well-type scintillation counter. All samples were counted in triplicate until at least 1,000 counts were recorded and were reported as counts per minute. Background radiation was determined prior to and after each series of samples analyzed and was subtracted from the experimental measurements. After the determination of radioactivity, the samples were dried in a hot-air oven at 95° to 100° C. for 24 hours; this allowed the concentration of ThO_2 to be expressed as counts per minute per gram of dry weight. Moreover, for valid comparisons between different experiments, the results were expressed as per cent uptake of the injected dose. Two preparations of the injected dose were counted along with each set of experimental samples.

In Series 1 the effects of adrenalectomy and of replacement treatment with graded amounts of cortisone were tested upon the uptake of colloidal thorium by splenic and hepatic tissues. Five groups of Long-Evans rats, each consisting of 10 animals, were used: Group A received no treatment; Groups B, C, and D received two subcutaneous doses daily, respectively, of 0.125, 0.5, and 1.5 mg. cortisone in 0.1 ml. physiologic saline for two days; Group E was injected with saline only. On the third day, animals in Group A were sham-operated; animals in Groups B, C, D, and E were adrenalectomized and continued on similar doses of hormone (Groups B to D) and saline (Group E) given five times over the subsequent 24 hours. Following this all animals were injected intravenously (via the jugular vein under light anesthesia) with 0.3 ml. thorotrast and were sacrificed by exsanguination six hours later. The spleens and livers were removed and analyzed chemically for thorium content.

Similarly, in Series 2, the effects of adrenalectomy and of replacement treatment with cortisone were tested upon the uptake of colloidal thorium by splenic and hepatic tissues. However, the thorium in these tissues was analyzed by radiochemical methods. Four groups of Wistar rats were used: Groups A and B, each consisting of 15 animals, received no treatment; Group C (10 rats) received two doses daily of 1.5 mg. cortisone in 0.05 ml. saline for two days; Group D (10 rats) received similar doses of saline only. On the third day animals of Group A were sham-operated; Groups B, C, and D were adrenalectomized and Groups C and D were continued on similar doses, respectively, of hormone and saline

given five times over the subsequent 24 hours. Following this, animals were injected intravenously with 0.3 ml. thorotrast and were sacrificed by exsanguination six hours later. Spleens and livers were removed and assayed for radioactivity.

In Series 3, the effects of hypophysectomy and of replacement treatment with various adrenal and hypophyseal hormones were tested upon the uptake of colloidal thorium by splenic and hepatic tissues. Six groups of Wistar rats were used at 24 to 28 days after hypophysectomy. Group A (10 rats) received no treatment; Groups B, C, D, E, and F (6 rats each) received daily subcutaneous injections, respectively, of 0.5 mg. ACTH in 0.1 ml. oil, 1 mg. GH in 0.1 ml. acid water, 1 mg. hydrocortisone in 0.1 ml. oil, 1 mg. GH plus 1 mg. hydrocortisone in 0.2 ml. volume, and 0.1 ml. of either acid water or oil. Three hours after injections on the seventh day, all animals were injected intravenously with 0.3 ml. thorotrast and were sacrificed by exsanguination 6 hours later. Spleens and livers were removed and assayed for radioactivity.

Since there was no statistical difference between the animals receiving either vehicle, these were pooled. Moreover, five normal intact rats were included in these experiments (Group G). They were not significantly different from, and are accordingly included in, Group 2A.

Data were analyzed by the analysis of variance, using Snedecor's F value as the test of significance.[7] Individual means were compared by use of the Least Significant Difference (LSD);

$$\text{LSD} = \sqrt{s^2 \left(\frac{n_1 + n_2}{n_1 n_2}\right)} \times t$$

where s^2 is the estimate of the error variance, n_1 and n_2 are the numbers of samples in each mean, and t is Student's "t" at the 1 per cent level of significance, based on the degrees of freedom in s^2. Any means differing by a value equal to or greater than the LSD are significantly different at the 1 per cent level. Unless otherwise indicated, all statements made in the text regarding differences between mean values are significant at the 1 per cent level.

RESULTS

Chemical analyses (Figs. 1 and 2) revealed that adrenalectomy resulted in a significantly decreased uptake of thorium by the spleens of female Long-Evans rats. All doses of cortisone employed increased the deposition of the metal in the splenic tissues of operated animals to normal levels. No dose of cortisone, however, was capable of increasing the uptake *above* normal. In addition, neither adrenalectomy nor replacement treatment with cortisone caused any significant change in the uptake of thorium in the liver.

Radiochemical analyses yielded similar results in male Wistar rats (Figs. 3 and 4). Specifically, adrenalectomy effected a significant decrease in the thorium concentration in the spleen, but not in the liver. Cortisone restored these quantities to normal levels in the spleen, but had no action in the liver.

Figs. 1 and 3 indicate that adrenalectomy resulted in approximately a 70 per cent decrease in the uptake of thorium by the spleen. This was true whether the results were expressed as milligrams of ThO_2 per gram dried tissue or as the per cent of the injected radioactivity per gram dried tissue.

Hypophysectomy, on the other hand, significantly increased the uptake of thorium by splenic and hepatic tissues (Figs. 5 and 6). Replacement treatment with ACTH, GH, hydrocortisone, or a combination of GH and hydrocortisone significantly diminished the concentration of thorium observed in the liver of the operated animals, but did not restore the values to normal. These hormones caused no significant changes in uptake by the spleen.

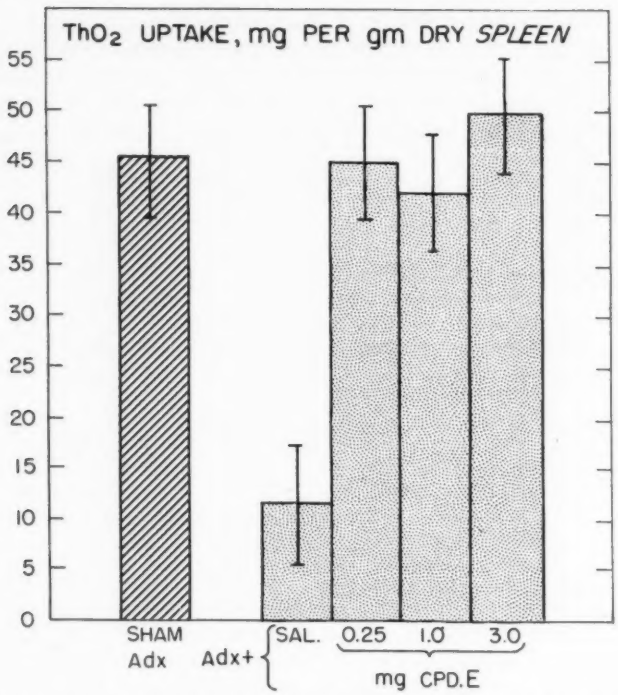

Fig. 1.—Mean uptake of ThO_2 by spleens of adrenalectomized and sham adrenalectomized rats, determined by chemical analyses. In this and subsequent figures, vertical lines through the tops of bars represent ± 1 standard error of the mean. See text for various experimental treatments employed.

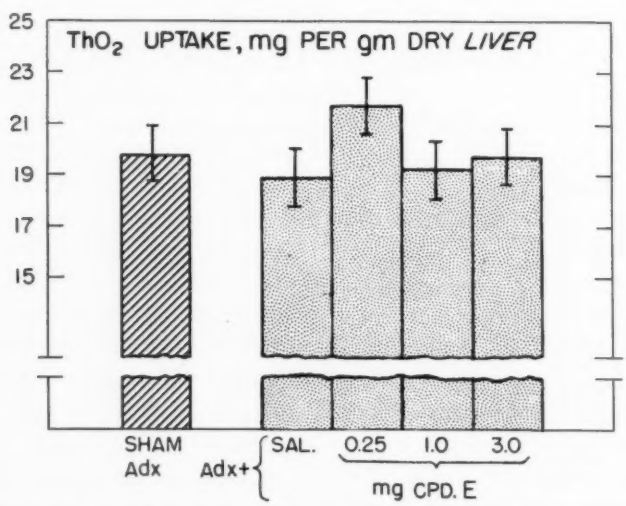

Fig. 2.—Mean uptake of ThO_2 by livers of adrenalectomized and sham adrenalectomized rats, determined by chemical analyses.

DISCUSSION

Both the chemical and radiochemical analyses of the present study reveal that adrenalectomy caused a significant diminution in the quantities of injected thorium accepted by the spleen. This confirms previous findings.[1-3]

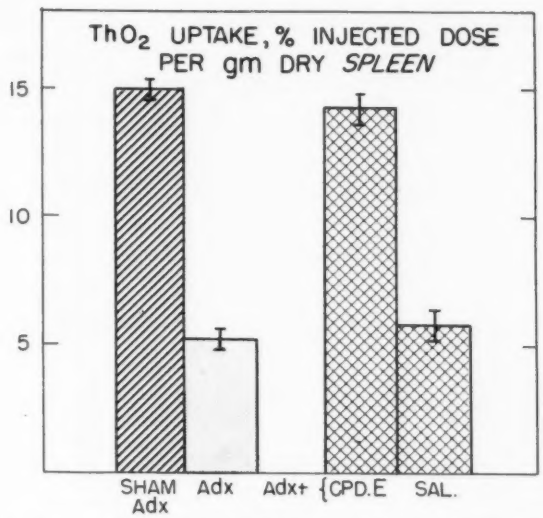

Fig. 3.—Mean per cent uptake of ThO_2 by spleens of adrenalectomized and sham adrenalectomized rats, determined by radiochemical analyses.

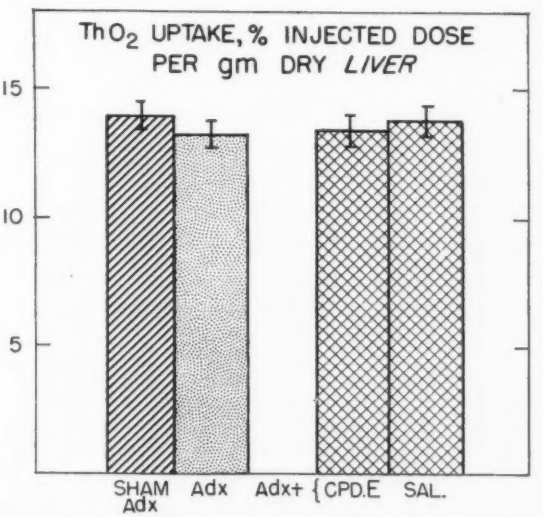

Fig. 4.—Mean per cent uptake of ThO_2 by livers of adrenalectomized and sham adrenalectomized rats, determined by radiochemical analyses.

In addition, it was demonstrated that cortisone administered to the adrenalectomized animal restored the ability of the spleen to accept normal quantities of these colloidal particles. Neither adrenalectomy nor cortisone treatment altered the uptake of thorium by the liver.

These findings are of especial interest in view of the recent emphasis placed on adrenocortical function in resistance to infection and intoxication. In general, the resistance to various stresses including bacterial infections has

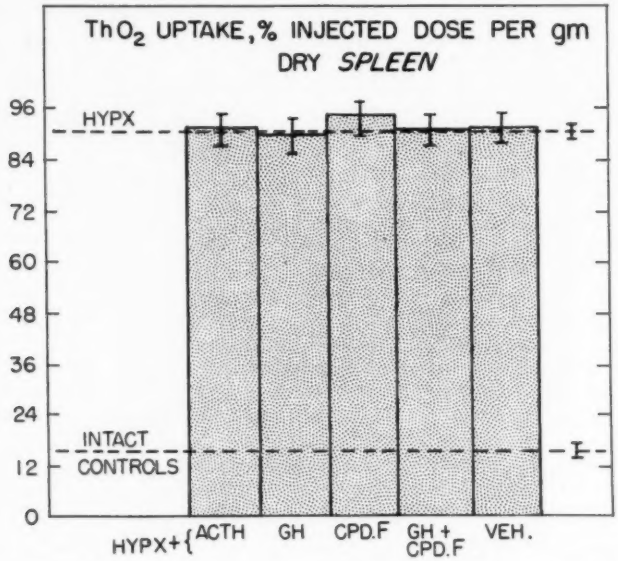

Fig. 5.—Mean per cent uptake of ThO_2 by spleens of hypophysectomized rats and intact controls.

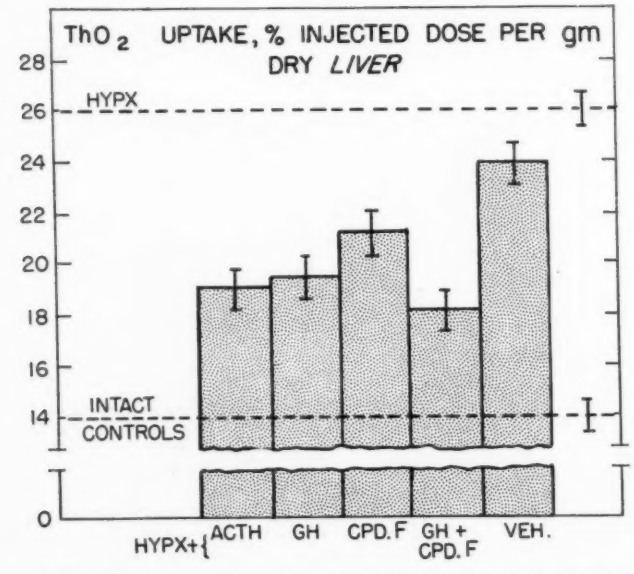

Fig. 6.—Mean per cent uptake of ThO_2 by livers of hypophysectomized rats and intact controls.

been shown to be decreased by adrenalectomy and restored to normal after replacement treatment with adrenocortical hormones.[8-11] Several other investigations have suggested that increased adrenal function or ACTH and

cortisone administration stimulated the phagocytosis of particulate matter. Thus an increased deposition within the spleen and liver of India ink,[12-14] tubercle bacilli,[13] lithium carmine,[15] carboxycellulose,[16] and silica particles,[17] and an increased removal from the blood stream of India ink[18] and tubercle bacilli[12] were noted with ACTH or cortisone administration or in states of adrenal hyperactivity. Histologically, increased production of macrophages in lymphoid structures was observed following adrenal cortical stimulation.[19, 20] An increased opsonic index[21] and a stimulated migration of macrophages in tissue cultures[22] was observed with the administration of certain cortical steroids. On the other hand, adrenal hormones may lower the resistance to various infecting agents.[23-26] In this regard, cortisone and ACTH were reported to depress the phagocytic activity of the reticuloendothelial cells toward India ink in mice,[27, 28] radioactive colloidal gold in rabbits,[29] radioactive colloidal chromic phosphate,[30, 31] and quartz particles in rats.[32] The phagocytic activity of polymorphonuclear leukocytes from rabbits[33-36] and from humans[37, 38] receiving cortisone or ACTH was also significantly retarded. Moreover, the digestive capacity of the reticuloendothelial cells for ingested microorganisms, including tubercle bacilli, pneumococci, streptococci, and red blood cells, was reduced following cortisone treatment.[12, 13, 26, 39-41] Along the same lines, the migration and growth of macrophages in tissue culture were suppressed by cortisone.[42-44]

Recently much attention has been given to hormonal influences on inflammatory reactions. Dougherty and Schneebeli,[45] for example, described significant inhibition by cortisone of polymorphonuclear leukocytic infiltration into areas of localized inflammation in sensitized and adrenalectomized mice. Rebuck and associates[46-48] reported a diminished phagocytic activity of leukocytes in experimental inflammation in humans treated with ACTH. This diminution of the phagocytic response has been confirmed by some,[49-56] but not by others. Thus, no effect of ACTH or cortisone was noted on the phagocytosis of India ink,[16, 57-59] caesium^{134}-coated glass microspheres,[60] radioactive gold particles,[61] or bacteria.[61, 62] In addition, the digestive capacity of phagocytes[63] and the growth of macrophages in tissue culture[64] were unaffected by these hormones.

The adrenal cortex is necessary in maintaining the constancy of the internal environment and is vital, therefore, in the resistance to all types of stress. Since the hormonal needs of the tissues may vary widely, the secretion of adrenocortical factors may be dependent upon their peripheral utilization. Indeed, dosages which are pharmacological under normal conditions are insufficient to maintain an animal in severe stress.[65, 66] Accordingly, experimental and therapeutic dosages of cortisone must be based on tissue requirements. In this respect, it was demonstrated that large doses of cortisone decreased the survival of intact and adrenalectomized animals subjected to the stress of experimental pneumococcal infection, whereas smaller doses of this hormone enhanced the host resistance to the infectious agent.[25, 67, 68] These

alterations may be interpreted, in part, in terms of changes in phagocytosis. A similar enhancing action of small doses of cortisone on the phagocytosis of staphylococci was recently reported.[69]

The present study indicates that hypophysectomy increased the deposition of thorium in both spleen and liver. Replacement treatment in the hypophysectomized rat with ACTH, GH, hydrocortisone, and a combination of GH and hydrocortisone significantly diminished the concentration of thorium in the liver of operated animals, but did not restore the values to normal. Similar results were obtained recently for India ink.[70] This phenomenon is somewhat difficult to understand in view of the depressive effects of adrenalectomy.* Nevertheless several explanations seem plausible. A low but measurable secretion of adrenal steroids persists after hypophysectomy.[72, 73] In the lowered metabolic state, characteristic of the hypophysectomized animal, this residue of activity may be sufficient to increase the phagocytic activity above normal. Moreover, the present studies indicate that replacement treatment in the hypophysectomized rat did not result in depression of phagocytosis. The heightened values in liver were lowered, but not even to normal levels. This concurs with the data of Rawls and associates.[70] In this regard, it is interesting to note that while Gordon and Katsh[1] found little change in the reticuloendothelial system following hypophysectomy, the administration of large amounts of adrenal cortical extract to the hypophysectomized rats stimulated phagocytosis. The possibility exists that the augmented phagocytic activity in the hypophysectomized animal is due to the continued production of aldosterone. This adrenal factor, whose secretion might be independent of the hypophysis,[72, 74, 75] was observed to stimulate the phagocytosis of destroyed eosinophiles by macrophages in the peritoneal fluid.[76] It is further possible that other types of compensatory mechanisms not directly related to the endocrine system are operating in the hypophysectomized animal.

SUMMARY

The influence of the adrenal and hypophysis upon the uptake of colloidal thorium dioxide by splenic and hepatic tissues was determined by both chemical and radiochemical analyses. Adrenalectomy resulted in a decreased uptake of thorium by the spleen. This action was prevented by the administration of cortisone. However, neither adrenalectomy nor replacement treatment with cortisone affected the accumulation of thorium within the liver. Hypophysectomy, on the other hand, increased the thorium concentration in both spleen and liver. This was prevented partially in the liver by ACTH, GH, hydrocortisone, and a combination of GH and hydrocortisone. These hormones had no effect upon the uptake of thorium by the spleen of the hypophysectomized rat. The relation of the endocrine system to phagocytosis is discussed.

*The reticuloendothelial system behaves differently in response to different particles. Thus, adrenalectomy results in increased uptake of Au^{198} (see Discussion, Reichard, Edelmann and Gordon[71]).

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STUDIES ON COPPER METABOLISM

XXII. Hemolytic Anemia in Chickens Induced by the Administration of Copper

A. Goldberg, M.B., M.R.C.P.(Lond.),* C. B. Williams, B.S., R. S. Jones, M.D., Mitsue Yanagita, B.S., G. E. Cartwright, M.D., and M. M. Wintrobe, M.D., Ph.D.
Salt Lake City, Utah

METALS such as colloidal silver, arsenic, and lead are recognized among the chemical agents which may cause a hemolytic anemia.[1] Copper is seldom included in this group, yet Albiston and associates[2] described a condition in Australian sheep called "enzootic jaundice" which was characterized by a hemolytic anemia and increased liver copper. They considered that the hemolytic anemia was due to a sudden mobilization of copper from the liver into the blood stream. Marston and Lee[3] were able to reproduce this state experimentally by feeding copper to sheep for 349 days or more. On the other hand, Flinn and Inouye,[4] and Eden[5] fed copper to rats and rabbits, respectively. Although increased levels of copper were found in the livers of the animals in both of these experiments, a hemolytic anemia was not observed.

The serum copper of the chicken is low.[6] It was considered, therefore, that this might prove to be a useful experimental animal in studying the role of copper in blood destruction. In the experiments to be described it will be shown that in a group of chickens given copper acetate for 2 to 5 weeks, 29 per cent of the animals developed a hemolytic anemia. In an attempt to determine whether the mechanism suggested for the anemia in sheep is applicable to that of the fowl, observations were made concerning the in vitro effect of copper salts on chicken, rabbit, and human erythrocytes. These studies have confirmed the reports of Lambin and associates,[7] and of Sautter and Lépine,[8] who found that the copper ion in low concentration causes agglutination and/or hemolysis of erythrocytes.

METHODS

Thirty adult white Leghorn hens, weighing 1.8 ± 0.25 kilograms, were fed commercial chicken pellets and water ad libitum. Copper acetate in gelatin capsules was given orally to each animal in increasing dosage as follows: 50 mg. copper (i.e., 159 mg. copper acetate) daily for 1 week, 75 mg. copper daily for a second week, then 100 mg. of copper daily until signs of anemia or toxicity appeared or death occurred. Weekly or more frequent

From the Departments of Medicine and Pathology, University of Utah College of Medicine, Salt Lake City, Utah.

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hematological and plasma copper determinations were carried out throughout the course of the experiment in Chickens 1 to 12, inclusive. Chickens 13 to 20 were sacrificed at intervals in order to obtain information concerning the copper content of the livers in the early period of copper administration. Thus, three chickens were sacrificed on Day 0, 3 on Day 7, and 2 on Day 21 of copper administration. Plasma copper determinations and erythrocyte fragility studies were done on these animals on the day of sacrifice. Hematological and plasma copper determinations were carried out on Chickens 21 to 30 only when they showed signs of anemia (pallor of comb) or toxicity (weakness and loss of weight). Fowls 22, 24, 27, and 30 died before such determinations were performed.

Preparations for the enumeration of reticulocytes were made by adding 5 drops of blood to 5 drops of a 1 per cent solution of brilliant cresyl blue in 1 per cent saline. After twenty minutes a thin smear was made and counterstained with Wright's stain. Five hundred erythrocytes were examined. Cells with five or more blue-staining particles were considered to be reticulocytes for the purpose of this study.

Osmotic saline fragility was measured by the method of Suess and associates[9] on blood obtained from four normal chickens and six chickens given copper acetate, one (No. 5) of which was anemic.

In order to test the effect of copper acetate on erythrocytes in vitro, a "copper fragility test" was carried out on blood from 10 normal chickens and 9 copper-fed chickens, two of which (Nos. 5 and 28) were anemic. The test was performed as follows: 20 cubic millimeters of heparinized chicken blood was added to 10 ml. of a copper-saline (0.9 per cent) solution. The copper concentration in the latter ranged from 5 to 500 mcg. per cent of copper as copper acetate. These levels of copper were insufficient to change the isotonicity of the normal saline. The tubes were incubated at 37° C. for one hour and then examined microscopically for agglutination. The tubes were then centrifuged at 1,000 r.p.m. for 5 minutes and examined visually for hemolysis.

Samples of plasma were examined spectroscopically for oxyhemoglobin, methemoglobin and methemalbumin. Determinations of plasma bilirubin,[10] blood methemoglobin,[11] and plasma hemoglobin[12] were made on all sacrificed animals.

Plasma and tissue copper determinations were done by the method of Gubler and associates,[13] and Lahey and associates,[14] respectively.

Microsections of the livers were stained with hematoxylin and eosin, with Perls' reaction for iron[15] and with Mallory's stain for copper.[16]

RESULTS

General Observations.—All birds lost weight progressively while receiving copper (Tables I and II). After two to six weeks of copper administration the birds became weak, anorexic, and lethargic. They assumed a hunched-up posture and were unresponsive to stimuli. In those birds which became anemic the comb became very pale. All of the chickens, with the exception of No. 7, died within 1 to 10 days after the first appearance of signs of toxicity or anemia, despite the fact that copper administration was stopped when these signs were first detected. Chicken 7 recovered and the hemoglobin concentration returned to normal 40 days later.

Hematologic Observations.—Eight of the 23 chickens tested hematologically developed anemia (Table I). The anemia appeared concomitantly with clinical signs of toxicity. The course of one such bird is shown in detail in Fig. 1.

In five (Nos. 1, 7, 8, 11, and 21) of the eight anemic birds there was a significant reduction in the mean corpuscular hemoglobin concentration. Examination of the smears of these chickens revealed polychromatophilia, anisocytosis, macrocystosis, and hyperchromia (Fig. 2). In the other three birds

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TABLE I. SUMMARY OF RESULTS IN ANEMIC CHICKENS

CHICKEN NO.	AVERAGE DOSE COPPER (MG./KG./DAY)	INITIAL WT. (KG.)	FINAL WT. (KG.)	ONSET OF ANEMIA (DAY)	DEATH (DAY)	HEMATOLOGICAL DATA (AT HEIGHT OF ANEMIA) V.P.R.C. (ML./100 ML.)	Hgb. (GM. %)	M.C.H.C. (%)	RETIC. (%)	II UNITS	MAXIMUM PLASMA COPPER (MCG. %)	TOTAL LIVER COPPER (MG.)
*Normal (mean)						34.3 ± 2.0	10.4 ± 0.6	31 ± 0.8	6 ± 2.7	7.5 ± 2.2	33 ± 10.5	0.18
Normal (range)						31-37	9.5-11.2	30-32	2-10	2-10	21-54	0.11-0.29
1	46	1.7	0.8	24	30	16.0	3.9	24	48	2	201	22.9
5	43	2.0	1.3	35	37	26.0	8.0	31	8	2	45	35.3
7	50	1.6	0.9	21	–	22.0	5.2	24	80	2	54	–
8	38	1.9	1.2	14	16	30.0	8.2	27	15	2	75	21.8
11	32	2.3	1.3	21	29	31.0	7.2	23	91	2	87	41.2
21	41	2.0	1.1	28	29	22.0	5.7	26	32	2	393	28.8
28	52	1.6	1.0	30	40	19.5	6.2	32	15	5	90	32.6
29	55	1.4	0.9	23	26	18.0	5.5	31	6	2	129	45.2

V.P.R.C., volume of packed red cells; Hgb., hemoglobin; M.C.H.C., mean corpuscular hemoglobin concentration; retic., reticulocyte count.
*Normal hematological values refer to means ± standard deviation of values in Chickens 1 to 12 at Day O. Normal total liver copper refers to mean of values of Chickens 13, 14, and 15 sacrificed on Day O.

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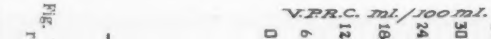

(Nos. 5, 28, and 29) the anemia was normochromic and the morphology of the erythrocytes was not remarkable. Reticulocytosis, in some cases marked, occurred in six of the birds.

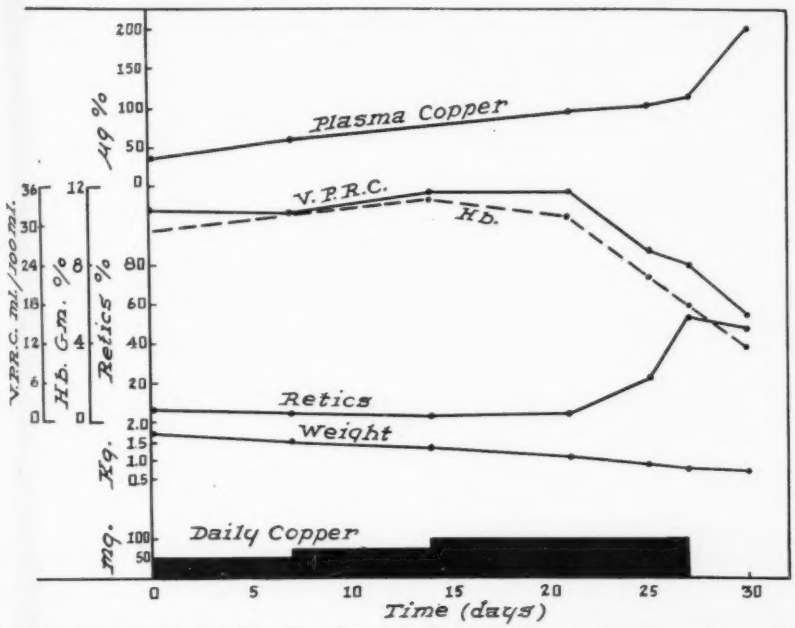

Fig. 1.—Effect of the administration of copper acetate on plasma copper, volume of packed red cells (V.P.R.C.), hemoglobin (Hgb.), reticulocytes, and body weight of a chicken.

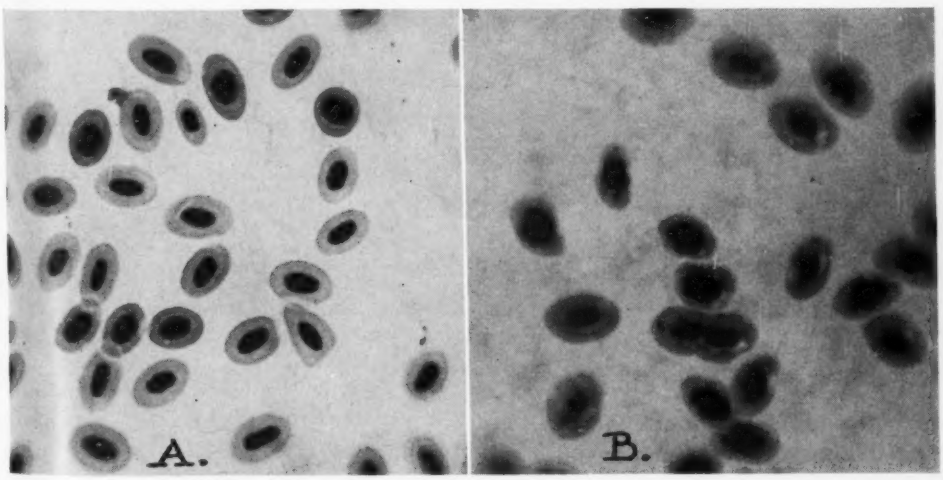

Fig. 2.—Blood smears stained with brilliant cresyl blue and Wright's stain. *A*, Blood of a normal chicken. *B*, Blood of an anemic chicken (No. 7).

Fifteen of the birds showed no hematologic abnormality (Table II), with the exception of a reticulocytosis of 18 per cent in one bird (No. 25).

There was no elevation of the icterus index in any of the birds. Total plasma bilirubin was measured in three control birds and in 11 birds given

TABLE II. SUMMARY OF RESULTS IN NONANEMIC CHICKENS

CHICKEN NO.	AVERAGE DOSE COPPER (MG./KG./DAY)	INITIAL WT. (KG.)	FINAL WT. (KG.)	DEATH (DAYS)	HEMATOLOGICAL DATA (AT OR JUST PRIOR TO DEATH) V.P.R.C. (ML./100 ML.)	Hgb. (GM. %)	M.C.H.C. (%)	RETIC. (%)	II UNITS	MAXIMUM PLASMA COPPER (MCG. %)	TOTAL LIVER COPPER (MG.)
*Normal (mean)					34.4 ± 2.0	10.4 ± 0.6	31 ± 0.8	6 ± 2.7	7.5 ± 2.2	33 ± 10.5	0.18
Normal (range)					31-37	9.5-11.2	30-32	2-10	2-10	21-54	0.11-0.29
2	50	1.6	0.8	27	36.5	10.9	30	11	2	126	39.7
3	48	1.7	0.9	28	34.0	10.2	30	5	2	102	40.1
4	43	1.8	1.1	24	36.0	10.8	30	9	2	108	45.1
6	55	1.4	1.0	27	35.0	11.0	31	2	2	51	21.5
9	44	1.8	1.0	26	48.5	14.3	30	11	2	762	21.2
10	34	2.4	1.8	28	41.5	13.1	31	2	2	63	28.0
12	43	1.8	1.4	24	43.5	13.3	31	11	2	597	12.3
16	24	2.1	1.8	7	39.0	11.4	29	6	2	15	4.5
17	29	1.7	1.6	7	38.5	11.4	30	1	2	24	2.5
18	31	1.6	1.5	7	37.0	10.9	29	2	2	33	3.8
19	39	1.9	1.4	21	37.5	11.4	30	3	2	36	19.7
20	39	1.9	1.1	21	37.5	11.0	29	2	2	54	10.3
23	44	2.0	1.0	42	37.0	11.2	30	11	2	39	32.3
25	46	1.8	1.1	30	34.0	10.8	32	18	2	156	29.0
26	47	1.8	1.2	35	34.5	10.5	30	9	2	69	30.1

For key to abbreviations see Table I.

*Normal hematological values refer to means ± standard deviation of values in Chickens 1 to 12 at Day O. Normal total liver copper refers to mean of values of Chickens 13, 14, and 15 sacrificed on Day O.

copper. The mean value for the control group was 0.06 mg. per cent (0.0 to 0.18 mg.) and for the experimental group, 0.18 mg. per cent (0.0 to 0.44 mg.). There was no significant difference between the groups and no difference was noted between the anemic and the nonanemic chickens. Hemoglobin, methemoglobin, and methemalbumin were not found in the plasma of any of the birds. Abnormal blood pigments were not noted in the erythrocytes.

Plasma Copper.—The mean maximal plasma copper value in the 12 control chickens was 33 ± 10.5 mcg. per cent. The plasma copper level increased above normal in 14 of the 23 birds (Tables I and II). The mean maximal value for the birds with anemia was 134 mcg. per cent (45 to 393 mcg.), and for the chickens without anemia, 149 mcg. per cent (15 to 762 mcg.).

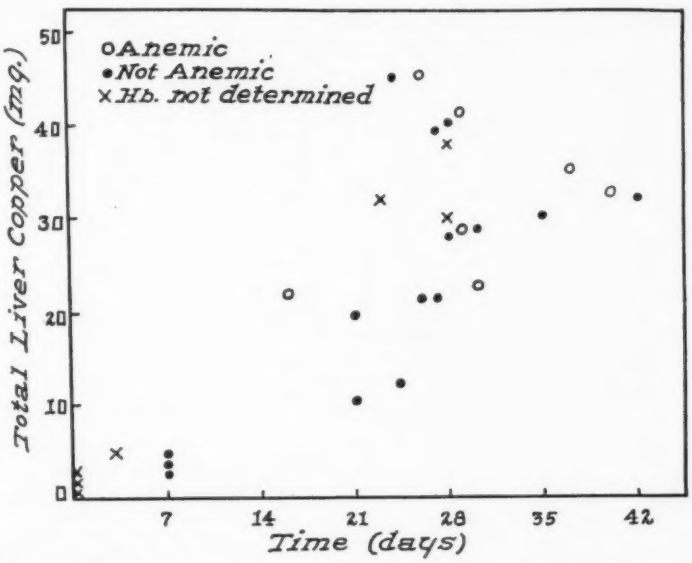

Fig. 3.—Relation of total liver copper to the period of copper administration.

Liver Copper.—The liver copper values are presented in Tables I and II and are plotted against time in Fig. 3. There was a progressive increase in the amount of copper in the liver. The mean content of copper in the liver for all birds given copper, irrespective of the period of time, was 26.2 mg., or 145 times that of the control mean. The amount of copper in the liver of the birds with anemia was no greater than the amount in the liver of the nonanemic birds given copper for a comparable period of time (Fig. 3). There was no correlation between the amount of copper in the liver and the degree of anemia (Table I).

Histopathology of Livers.—Erythrophagocytosis and hemosiderosis were noted within the Kupffer cells lining the sinusoids of most of the copper-fed animals (Table III). These findings were not observed in the livers from control birds.

The erythrophagocytosis was manifested by disintegrated erythrocytes, agglutinated erythrocytes, and clusters of pyknotic erythrocytic nuclei within

the Kupffer cells. Agglutinated erythrocytes were also observed in the sinusoids without detectable relation to Kupffer cells (Fig. 4).

There was a rough correlation between the degree of erythrophagocytosis and hemosiderosis. Iron was found only within Kupffer cells. In some animals it was scattered uniformly throughout, in others the iron was clustered in adjacent Kupffer cells.

Mallory's stain for copper was positive in the animals fed copper. The blue granules, representative of copper, were noted predominantly within Kupffer cells.

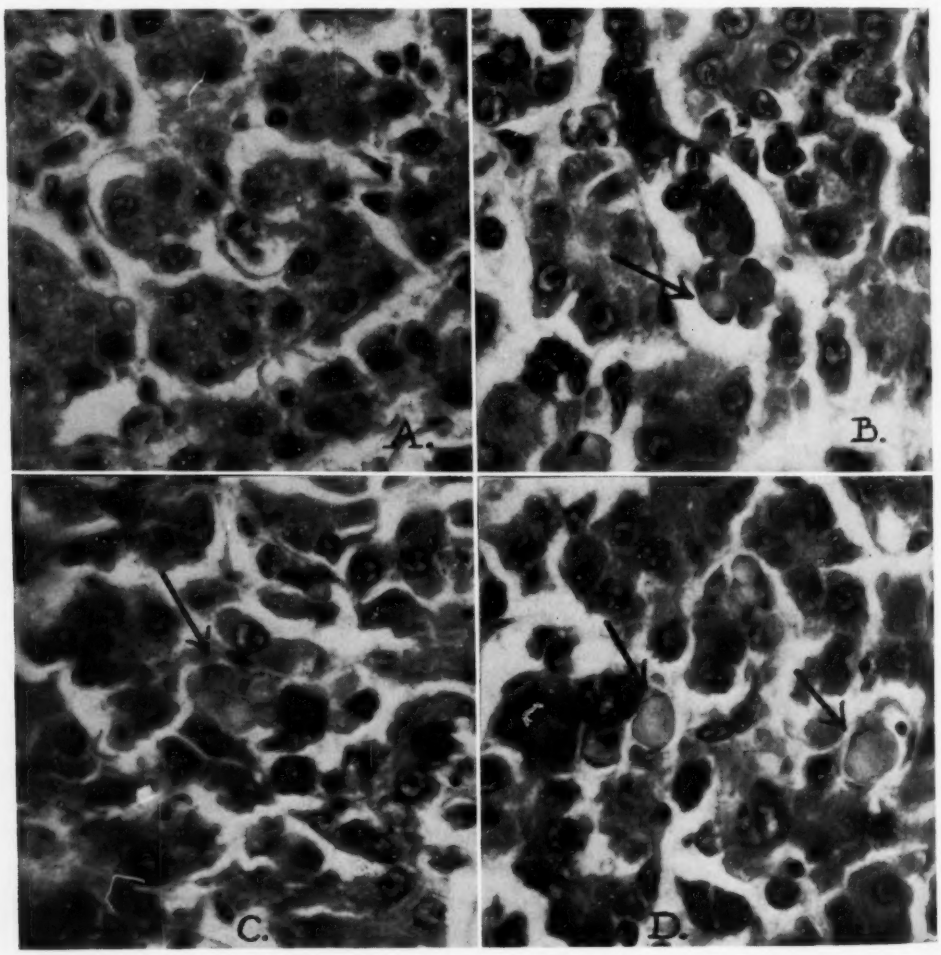

Fig. 4.—Sections of liver stained with hematoxylin and eosin and with Perls' stain for iron. *A*, Normal liver. Note the absence of erythrophagocytosis and hemosiderosis. *B*, *C*, and *D*, Sections of livers from chickens fed copper. In *B* the erythrocytes and hemosiderin are in sinusoidol Kupffer cells. In *C* and *D*, note phagocytosis of erythrocytes by Kupffer cells and iron-containing pigment in adjacent Kupffer cells.

Osmotic Fragility Studies.—In the 4 control birds, hemolysis began at 0.50 to 0.52 per cent saline and was complete at 0.32 per cent. In the 6

chickens given copper, hemolysis began at 0.50 to 0.56 per cent saline and was complete at 0.32 to 0.36 per cent. Representative curves for one control chicken and one copper-fed anemic chicken (No. 5) are given in Fig. 5.

TABLE III. SUMMARY OF THE HISTOLOGIC FINDINGS IN THE LIVER

GROUP	CHICKEN NO.	IRON IN KUPFFER CELLS	ERYTHROPHAGOCYTOSIS
Control	13	0	0
	14	0	0
	15	0	0
Copper-fed anemic	1	++++	++++
	5	+	±
	8	+	±
	11	+	±
	21	++	++
	28	++	++++
	29	++	+
Copper-fed nonanemic	2	+++	±
	3	++	±
	4	±	0
	6	±	++
	9	+++	±
	10	+	+
	12	+	+
	19	+	±
	20	±	0
	23	++	+
	25	++	+
	26	+	+

TABLE IV. RESULTS OF COPPER FRAGILITY STUDIES ON NORMAL AND COPPER-FED CHICKENS

GROUP	NO. OF CHICKENS	MINIMUM CONC. OF Cu PRODUCING HEMOLYSIS (MCG./100 ML.)	MINIMUM CONC. OF Cu PRODUCING AGGLUTINATION (MCG./100 ML.)
Normal chickens	10	75 (50-100)	200 (100-250)
Chickens fed copper	9	100 (40-175)	150 (100-175)

Copper Fragility Studies.—The results are presented in Table IV. In the higher concentrations of copper salts, hemoglobin was precipitated out of solution. For this reason complete hemolysis curves could not be obtained and the results are presented as the minimum concentration of copper at which hemolysis was noted.

Agglutination of erythrocytes was also observed. This phenomenon invariably began at higher concentrations than did hemolysis. Agglutination and hemolysis were independent of the pH change induced by the copper salt. Copper sulfate, as well as copper acetate, produced agglutination and hemolysis of erythrocytes at identical concentrations of copper. There was no significant difference in the copper fragility tests of normal chicken erythrocytes and the erythrocytes from either anemic or nonanemic chickens receiving large amounts of copper.

In order to determine if copper bound to serum proteins had the same effect as copper acetate in saline, increasing concentrations of copper up to

5,000 mcg. per 100 ml. were added to normal chicken serum and also to serum obtained from a chicken receiving large doses of copper. No agglutination or hemolysis was observed in either case.

Copper fragility studies were also carried out on human and rabbit erythrocytes suspended in 0.9 per cent saline. Normal human erythrocytes (3 experiments) first began to hemolyze at concentrations of copper of 75 mcg. per cent, while agglutination began at 83 mcg. per cent (75 to 100 mcg.). Normal rabbit cells (4 experiments) began to hemolyze at a concentration of copper of 64 mcg. per cent (40 to 100 mcg.). Agglutination of rabbit erythrocytes was not noted with copper concentrations up to 500 mcg. per cent.

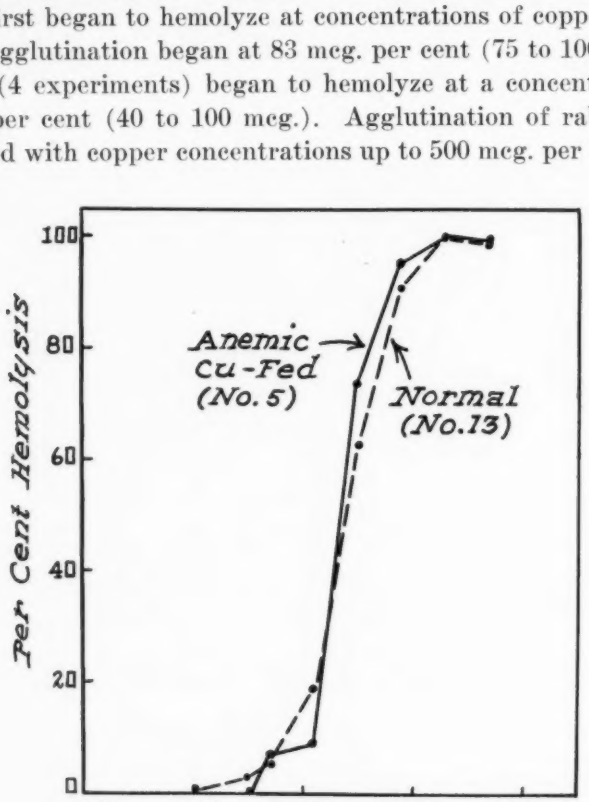

Fig. 5.—Osmotic saline fragility curves.

DISCUSSION

The administration of copper acetate was associated with the development of anemia in about one-third of the birds. Why two-thirds of the chickens developed evidence of toxicity without developing anemia is not understood. This difference in response cannot be explained by differences in the concentration of copper in the plasma or liver.

In unpublished experiments we have observed anemia in chickens following the administration of copper sulfate. This suggests that the effect is independent of the anion.

The combination of anemia with reticulocytosis, in the absence of evidence of blood loss, suggests that the anemia was due to a hemolytic process.

Why two of the anemic birds failed to develop reticulocytosis is not explained. The occurrence of a reticulocytosis in one bird without anemia could be due to a "compensated" hemolytic process.

It is interesting to speculate on the pathogenesis of the anemia. A mechanism envisaged by Albiston and associates[2] to account for the enzootic jaundice in sheep was that copper was suddenly mobilized from the liver to the plasma and that the excessive quantities of copper in the plasma resulted in intravascular hemolysis. There is no evidence that this occurred in the chickens. Although the plasma copper level was frequently elevated, an abrupt mobilization of copper into the plasma was not observed and there was no evidence of intravascular hemolysis. The plasma copper values were as high in the nonanemic birds as in the anemic ones. Finally, as demonstrated by the in vitro studies, when copper was added to serum even in concentrations up to 5,000 meg. per cent, neither agglutination nor hemolysis occurred. This was apparently due to the fact that the copper was rapidly and completely bound to the serum proteins. Since the plasma copper levels of the anemic birds were greatly below 5,000 meg. per cent and since we have shown previously[17, 18] that copper added to plasma either in vivo or in vitro is not in a dialyzable state, it is likely that a different mechanism must account for the anemia.

Another possible explanation for the anemia is that the concentration of copper in the red cells was increased and that this resulted in increased susceptibility of the cells to destruction. Although the concentration of copper in the erythrocytes was not measured in this study, from previous studies in other species[17] it seems likely that an increase in erythrocyte copper occurred under these conditions. However, there was no increase in the saline osmotic fragility of red cells from an anemic chicken as compared with that of a normal chicken and the susceptibility of such cells to hemolysis in vitro by copper added to saline was not increased.

Albiston and associates[2] have suggested that the main destruction of blood in the hemolytic disease of sheep may have occurred in the liver sinusoids. The finding of erythrophagocytosis in the livers of the chickens is quite compatible with this concept. The concentration of copper in the livers of the birds was greatly increased and it is quite possible that exposure of the erythrocytes to copper in the liver resulted in hemolysis.

Whether or not the observations in chickens have any relation to human disease cannot be stated. The studies indicate that human erythrocytes are also susceptible to the in vitro effects of copper. Likewise, it has been observed that the concentration of copper in the liver is increased in such conditions as cirrhosis of the liver,[19] malignant disease,[19] and Wilson's disease.[20] It is possible that the mild hemolytic process observed in these conditions[20-22] is related to the excessive deposition of copper in the tissues.

The in vitro studies on the hemolysis of erythrocytes by copper emphasize the experience of Sautter and Lépine,[8] that in agglutination tests of erythrocytes care should be taken that the apparatus and reagents are copper-free.

SUMMARY

1. Thirty adult chickens were given increasing amounts of copper acetate orally to the point of toxicity.

2. An anemia associated with reticulocytosis developed in 8 of 23 birds. The remainder died without evidence of anemia.

3. It is suggested that erythrocytes are destroyed in the liver as a consequence of exposure to excessive amounts of copper in that organ.

4. Copper in low concentrations caused agglutination and/or hemolysis when incubated with chicken, rabbit, or human erythrocytes suspended in saline. Copper failed to cause such effects, even at high concentrations, when erythrocytes were suspended in serum.

We are indebted to Mr. George Trappett and Misses Marilyn Jacobsen, Doris Kurth, Megan Scott, and Gwen Trappett for technical assistance.

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RADIOACTIVE (I^{131}-TAGGED) ROSE-BENGAL LIVER FUNCTION TEST

CHARLES H. BROWN, M.D., AND OTTO GLASSER, PH.D.
CLEVELAND, OHIO

INTRODUCTION

WITH the production of a radioactive Rose-Bengal (I^{131}-tagged) solution,* we became interested in its use as a test in the diagnosis of hepatobiliary disease. The material is injected intravenously, and a scintillation counter is directed over the liver to record the radioactivity as the solution is absorbed and subsequently excreted by the liver. The purpose of this paper is to report the results of this test in 104 patients with various clinical diagnoses.

Taplin, Meredith, and Kade[1] determined liver "uptake" and "excretion" curves by directing a gamma ray counter over the liver after radioactive Rose-Bengal solution had been administered. Experimentally, they found that in normal rabbits a rapid and high uptake was followed by rapid excretion; in rabbits 24 hours after carbon tetrachloride poisoning, the uptake was not as high nor as rapid and excretion was much slower; and in rabbits with common duct ligation there was a rapid and high uptake but no excretion. They found that patients with liver disease had decreased but more prolonged uptake and delayed excretion. These workers suggested that this test might be of help in the differentiation of obstructive from hepatic jaundice, and they reported: "It helps to differentiate primary hepatic cell dysfunction from obstructive biliary tract disease."[2]

Rosenberg, Lee, and Martignoni[3] reported the results of the radioactive Rose-Bengal test in 41 patients. They[4] now have used the test in approximately 100 patients and, in contrast to the report of Taplin and associates,[2] have found the test to be of no value in differentiating obstructive from hepatic jaundice.

Mendeloff[5] observed that the fluorescence of intravenously administered radioactive Rose-Bengal solution appeared only in the hepatic polygonal cells, and was not apparent in the Kupffer's cells. This suggests that the removal of the solution is normally a function of the hepatic parenchymal cells rather than of the Kupffer's cells. Mendeloff and Ingelfinger[6] also reported that the hepatic uptake of the Rose-Bengal solution was slowed or blocked by simultaneously administered bromsulfalein.

From the Departments of Gastroenterology and of Biophysics, The Cleveland Clinic Foundation, and The Frank E. Bunts Educational Institute, Cleveland, Ohio.

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*The radioactive material used in this investigation was obtained from the Abbott Laboratories, North Chicago, Ill., on authorization from the Isotopes Division, U.S. Atomic Energy Commission.

MATERIAL AND METHOD

In the present study of 104 patients, 25 μc of the radioactive (I^{131}-tagged) Rose-Bengal solution was intravenously administered, and a collimated scintillation detector pointed over the liver (Fig. 1) and directed away from the gallbladder area. The lead diaphragm of the collimator has a diameter of 2½ cm. and extends 7 cm. beyond the front of the detector tube. The scintillation tube is connected with a Nuclear-Chicago Isotron count rate meter and the radioactivity is recorded in counts per minute for the next 60 to 75 minutes. The instrument also contains an Esterline-Angus recording instrument which provides a parallel indication of the counts per minute and also permits making a permanent record over any desired length of time. In 24 of the 104 patients, additional scintillation counts were made in 4 and in 24 hours; in 10, the surface of the liver was scanned with the same scintillation detector by performing a series of localization counts over the same area; and in 15, the radioactivity in 24-hour urinary specimens was studied. The patients were not fasting.

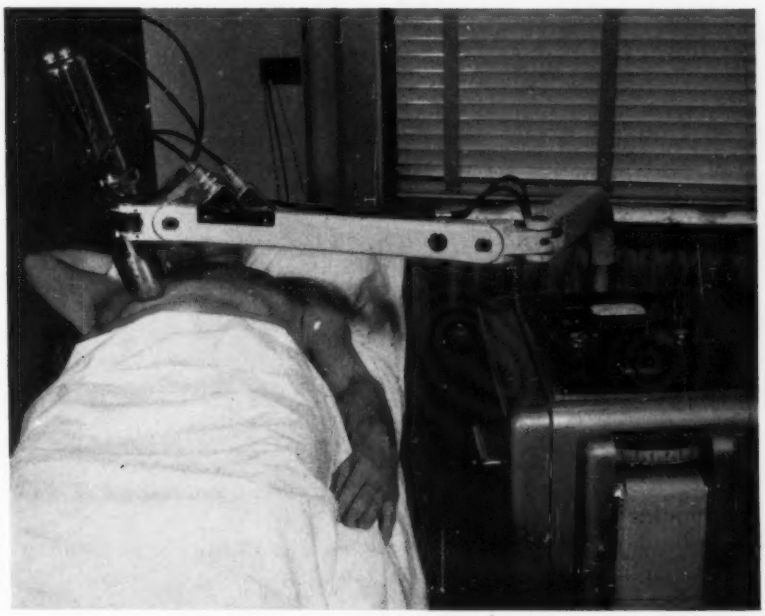

Fig. 1.—Positioning of scintillation counter over liver.

Thirty-three of the 104 patients had normal livers and the remaining 71 had one of the following conditions: cirrhosis, obstruction of the common bile duct, infectious hepatitis, or chlorpromazine jaundice. Before the Rose-Bengal test, most of the patients had had a battery of liver function tests consisting of cephalin flocculation, thymol turbidity, albumin-globulin ratio, alkaline phosphatase, and, in patients who were not jaundiced, the bromsulfalein liver function tests. In addition, 40 patients had had needle biopsy of the liver, and the pathologic diagnosis was confirmed in 20 other patients by operation after the Rose-Bengal test had been done. The patients with liver disease had extensive liver damage and were not "borderline" cases.

RESULTS

Uptake and excretion curves were obtained on 33 persons who had no evidence of liver disease. These curves are shown in Fig. 2. Initially there

is a rapid high uptake of the radioactive solution, in 30 minutes the curve levels out, and then the uptake slowly decreases as excretion occurs. The mean uptake in 15 minutes was 2.1 thousand counts per minute plus or minus the standard deviation of 0.7. (These figures* will be recorded subsequently simply as the mean plus or minus the standard deviation in thousand counts per minute.) In 60 minutes the mean was 2.7 ± 0.8. A count in four hours in eight patients showed that considerable excretion had occurred; the count had decreased to 1.2 ± 0.3. In 24 hours in the same eight patients the count was 0.6 ± 0.4, showing that further excretion had occurred. These figures are depicted graphically in Chart I.

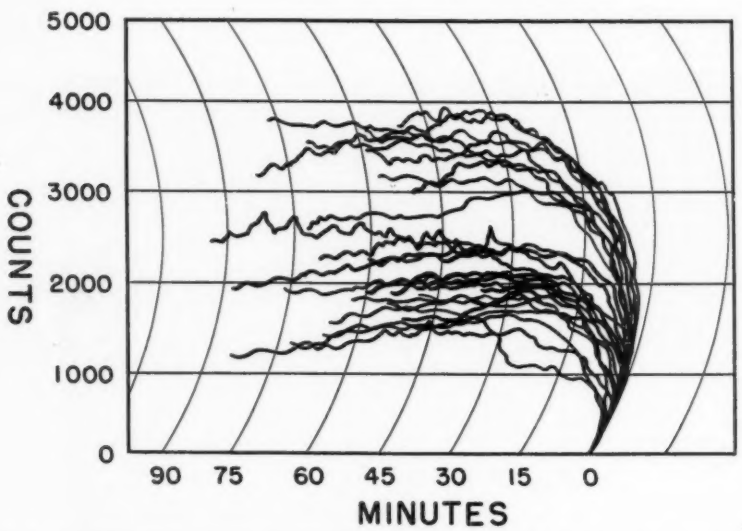

Fig 2.—Uptake and excretion curves of 33 persons showing no evidence of liver disease.

In 27 patients with Laennec's cirrhosis, the uptake was not as rapid and was more sustained (Fig. 3), and within the test period of 60 to 75 minutes, no excretion occurred. The mean uptake in 15 minutes was 0.9 ± 0.5 and in 60 minutes 1.2 ± 0.6. The uptake continued for a prolonged period, and excretion was delayed; as can be seen from the fact that in eight patients, counts at the end of four hours (1.6 ± 0.4) were higher than those at the end of 60 minutes. In 24 hours in these same eight patients the counts had decreased to 0.4 ± 0.4, indicating excretion of the radioactive solution by the liver.

Statistically significant differences were obtained between the normal and the cirrhotic group in the 15- and 60-minute and in the 4-hour counts; the T scores† were, respectively, 2.44, 5.9, and 2.63. There was no significant difference in the 24-hour counts.

*The statistical figures are presented as a trend only. N in each group was small, and there was considerable variability as can be seen from the standard deviations, both of which made the statistical figures less reliable.

†T scores computed by the formula[7]:

$$T = \frac{Mn_1 - Mn_2}{\sigma\, M_1 - M_2}, \text{ where } \sigma\, M_1 - M_2 = \sqrt{\sigma^2\, Mn_1 + \sigma^2\, Mn_2}\ ;\ \sigma\, M = \frac{\sigma}{\sqrt{N}}.$$

In 21 patients with obstructive jaundice, there was a slow prolonged uptake and delayed excretion (Fig. 4), producing curves similar to those in patients with Laennec's cirrhosis. No appreciable excretion was apparent in 60 minutes. In 15 minutes the mean was 1.0 ± 0.3, in 60 minutes 1.3 ± 0.4.

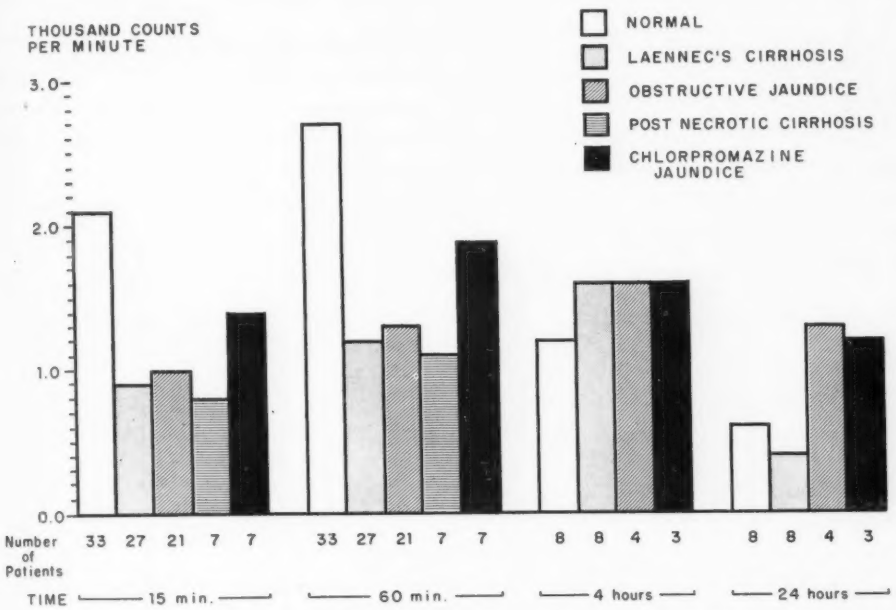
THOUSAND COUNTS PER MINUTE
3.0
2.0
1.0
0.0
NORMAL
LAENNEC'S CIRRHOSIS
OBSTRUCTIVE JAUNDICE
POST NECROTIC CIRRHOSIS
CHLORPROMAZINE JAUNDICE
Number of Patients 33 27 21 7 7 33 27 21 7 7 8 8 4 3 8 8 4 3
TIME 15 min. 60 min. 4 hours 24 hours

Chart I.—Radioactivity over the liver after I^{131} Rose-Bengal.

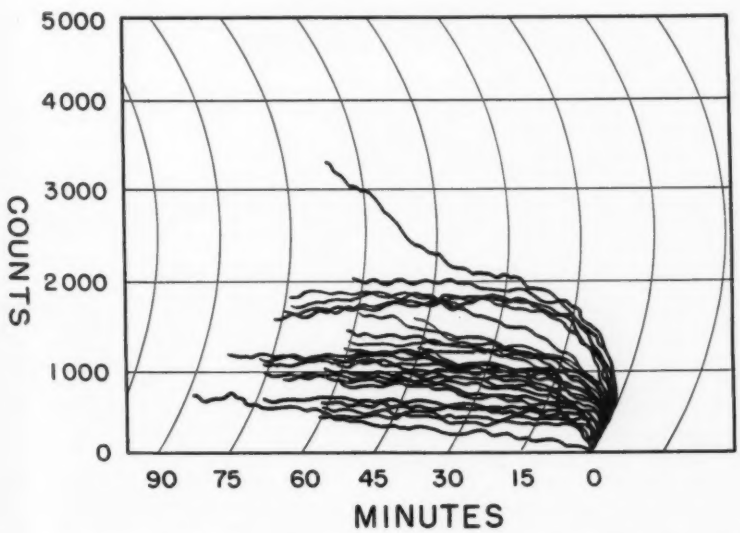

Fig. 3.—Uptake and excretion curves of 27 patients with Laennec's cirrhosis.

That the liver continued to take up the solution for a long period and that excretion was delayed are shown by the 4- and 24-hour counts in four patients, which were 1.6 ± 0.5 and 1.3 ± 0.2, respectively.

Statistically significant differences between the values in the normal group and those in the patients with obstructive jaundice were obtained in the 15-minute, 60-minute, and 24-hour tests; the T scores were 7.9, 5.6, and 3.7, respectively. There was no statistically significant difference in the 4-hour test, the T score being 1.7.

A comparison then was made between the cirrhotic group and the obstructive jaundice group. No statistically significant differences were obtained in 15 and 60 minutes and in 4 hours, the T scores all being less than 1. Twenty-four hours after the administration of the dye, four patients with obstructive jaundice showed greater retention than did the other groups, the counts being 1.3 ± 0.2 as compared to 0.6 ± 0.4 (8 normals) and 0.4 ± 0.4 (8 patients with cirrhosis). With these few patients there was overlapping between the counts in obstructive jaundice and in the normal individuals. Originally we were

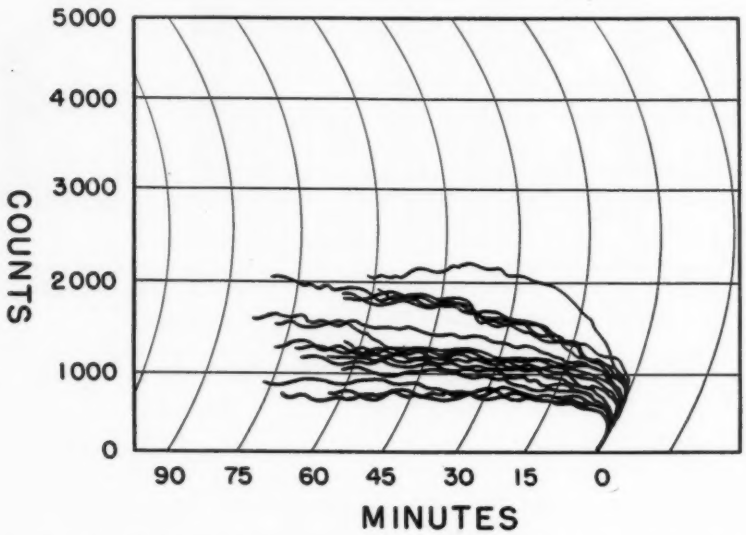

Fig. 4.—Uptake and excretion curves of 21 patients with obstructive jaundice.

interested in only 60- to 90-minute uptake-excretion curves. When these failed to demonstrate any significant difference between hepatic and obstructive jaundice, we increased the time of observation to 4 and 24 hours. Consequently the number of patients observed in 24 hours is still small. Further studies with the 24-hour count, and perhaps even longer test periods of 48 and 72 hours, might be of value.

It is obvious from the curves (Figs. 2, 3, 4) and from the statistical figures for isolated counts at 15 and 60 minutes and 4 hours, that in patients with Laennec's cirrhosis and in those with obstructive jaundice, the liver has delayed and decreased uptake and delayed excretion of the radioactive solution. In our studies, the test failed to differentiate Laennec's cirrhosis from obstructive jaundice in a 4-hour period. The uptake and excretion of the radioactive solution were impaired in obstructive jaundice just as they were in

Laennec's cirrhosis. On the other hand, Taplin and associates[1, 2] have reported the test of help in differentiating hepatic from obstructive jaundice. Rosenberg and Lee[4] who have used the test clinically in approximately 100 patients stated, "We agree with your comments concerning the failure to distinguish obstructive and hepatic jaundice."

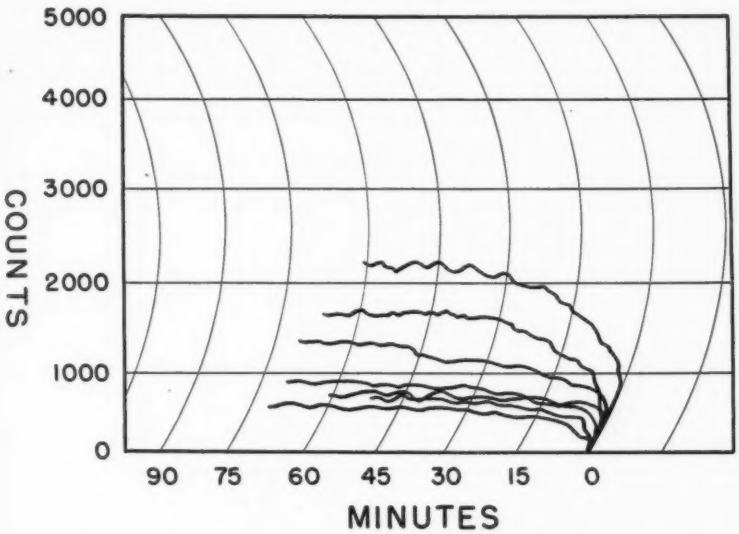

Fig. 5.—Uptake and excretion curves of 7 patients with postnecrotic cirrhosis.

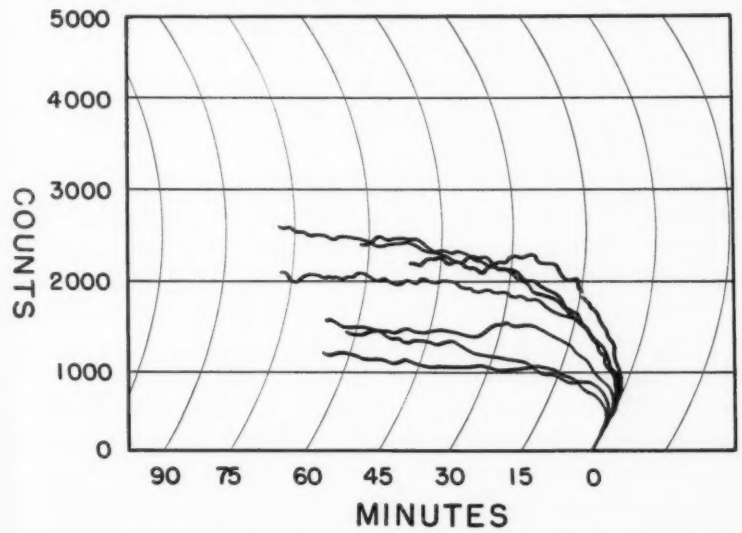

Fig. 6.—Uptake and excretion curves of 7 patients with chlorpromazine jaundice.

Seven patients with postnecrotic (or posthepatic) cirrhosis were studied and showed the delayed and decreased uptake and delayed excretions (Fig. 5) similar to, and in fact indistinguishable from, those of patients having Laennec's cirrhosis. In 15 minutes the uptake was 0.8 ± 0.4, and in 60 minutes 1.1 ± 0.6.

In the seven patients with chlorpromazine jaundice, the curves (Fig. 6) were identical to those in patients with Laennec's cirrhosis and obstructive jaundice. In 15 minutes the uptake was 1.4 ± 0.4, and in 60 minutes 1.9 ± 0.5. Excretion had not started in any of the patients in 60 minutes. Three patients in 4 hours had counts of 1.6 ± 0.1, and in 24 hours 1.2 ± 0.1.

Five patients (Fig. 7) with infectious (viral) hepatitis were studied. In four, the curves were similar to those in other conditions in which liver function is impaired. The fifth patient who had recovered from hepatitis had a normal curve, the count going to 2.7 in 15 minutes.

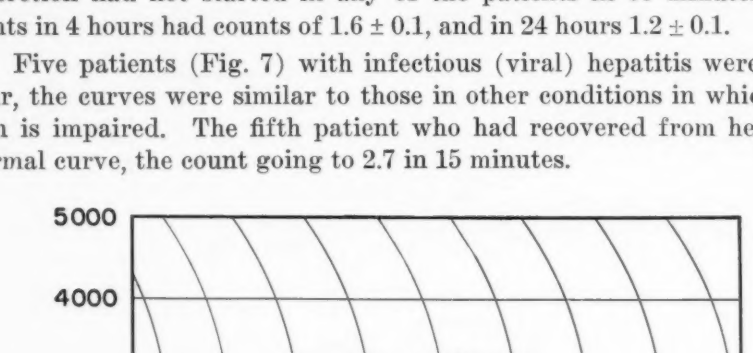

Fig. 7.—Uptake and excretion curves of 5 patients with infectious hepatitis.

Four patients with focal liver disease (hepatoma or metastatic malignancy) were studied. The curve in one showed poor uptake and impaired function, while those in the other three were normal. The entire area of the liver was scanned in these four patients, and in two, areas of a pronounced decrease in radioactivity were found. Over these areas the count would drop abruptly to 800, as compared with 2,000 counts per minute over the normal areas of the liver. The areas of decreased radioactivity corresponded with a large hepatoma, as determined by liver biopsy. Rosenberg and Lee[4] have reported a similar finding in a patient with a hepatoma.

DISCUSSION

The similarity of the uptake curves in patients with obstructive jaundice and in those with cirrhosis came as a surprise to us. Obstructive jaundice must result in decrease in function of the hepatic cell in taking up the radioactive Rose-Bengal solution. Mendeloff and Ingelfinger[6] found that the hepatic uptake of the Rose-Bengal solution was slowed by the simultaneous administration of bromsulfalein. This suggests that increased amounts of

bilirubin in the hepatic cell (such as occurs in obstructive jaundice) may also delay and impair the uptake of the Rose-Bengal solution. We believe it is this factor—the decrease in function of the hepatic cell in taking up the solution—that makes the Rose-Bengal test and probably the bromsulfalein test invalid and unreliable in the presence of obstructive jaundice.

The fact that this test does not differentiate hepatic from obstructive jaundice lessens its value as a liver function test. Since the material is radioactive, it deteriorates with time. The time required by the technician in obtaining the curves is a further drawback to the widespread clinical application of this test. Consequently, we do not believe that this test supplants or replaces the present liver function tests.

The I^{131}-tagged Rose-Bengal test has one advantage over other liver function tests commonly used, in that it detects areas of decreased radioactivity in the liver such as those that occur in cases of large hepatomas or metastatic malignancy. However, we invariably do a needle biopsy of the liver in patients suspected of having neoplasm of the liver, so that this one particular advantage is not clinically practical.

The test does give us further knowledge of the function of the hepatic cell. For example, the decreased function of the hepatic cell in taking up the radioactive Rose-Bengal solution in the presence of obstructive jaundice was surprising. Further studies with radioactive material should give us a greater knowledge of hepatic cell activity. Such studies should preferably use material other than radioactive dye solutions; since such other material may be more fruitful in differentiating hepatic from obstructive jaundice. Studies with heavy water, radioactive carbohydrates, amino acids, fats, and other radioactive material may provide further information concerning the metabolic functions of the liver.

In 15 of the 104 patients, 24-hour urinary specimens were collected and studied for radioactivity. Five of these 15 patients had no evidence of liver disease, four had Laennec's cirrhosis, two chlorpromazine jaundice, two obstructive jaundice, one focal liver disease, and one viral hepatitis. The activity in the urine was low, ranging from 0.6 (traces) to 4.0 μc. We were unable to correlate the degrees of radioactivity in the urine with the presence or absence of any of the various diseases.

SUMMARY

The radioactive (I^{131}-tagged) Rose-Bengal test was performed in 104 patients for a period of 60 to 75 minutes. In the 33 patients with normal livers there was a rapid uptake to high levels and excretion of the dye began in about 30 minutes. In the 27 patients with Laennec's cirrhosis there were a delayed and decreased uptake and delayed excretion. The differences in the test results between the cirrhotic group and the normal group were statistically significant.

In the 21 patients with obstructive jaundice the uptake curves were indistinguishable from those in patients with Laennec's cirrhosis. There were no statistically significant differences in the uptake curves of the patients

with obstructive jaundice and those with Laennec's cirrhosis; however, there were statistically significant differences in the uptake curves of the patients with normal livers and those of the patients with obstructive jaundice. We believe that in obstructive jaundice there is a decrease in the function of the hepatic cell in taking up the radioactive Rose-Bengal solution, and that in the presence of jaundice the dye tests of liver function are unreliable.

Counts at longer periods of time (24, 48, and 72 hours) than the 60 to 75 minutes used in the 104 patients might be of greater diagnostic value in jaundiced patients and might show greater retention in patients with obstructive jaundice as contrasted to those with normal livers and those with hepatic disease.

The seven patients with postnecrotic cirrhosis, the seven with chlorpromazine jaundice, and the five with infectious hepatitis showed impaired uptake of the solution similar to that in patients with Laennec's cirrhosis. Two patients with focal liver disease (hepatoma or metastatic carcinoma) had a pronounced decrease in radioactivity over the tumor, as compared with the rest of their liver.

Further studies of the liver with other radioactive material, preferably not dyes, are clearly indicated and should give us further information concerning the metabolic functions of the liver.

We are indebted to Miss Helen M. Bliss for technical assistance in this study. We are also indebted to Mr. Byron Svetlik who made the statistical calculations.

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LABORATORY METHODS

A SIMPLIFIED BLUE TETRAZOLIUM REACTION

RICHARD O. RECKNAGEL, PH.D.,* AND MARILYN LITTERIA, B.S.**
CLEVELAND, OHIO

VARIOUS forms of the blue tetrazolium reaction have been used for the quantitative estimation of adrenocortical steroids.[1-4] In the course of studies on the metabolism of adrenocortical steroids by enzyme systems of hepatic origin, the procedure of Weichselbaum and Margraf[3] adapted to a 3.0 ml. final volume was used to follow steroid disappearance. However, in our hands the method was unpredictable. The main difficulties were poor replication and marked variation in the degree of color development from day to day. The precautions as to cleanliness of glassware recommended by Weichselbaum and Margraf were followed and did not resolve the difficulties. The variability was finally traced to the various samples of tetramethylammonium hydroxide used. All tetramethylammonium hydroxide samples were products of Eastman Organic Chemicals. Three aged tetramethylammonium hydroxide preparations gave reproducible results. However, in two cases, freshly opened bottles gave aberrant results, and in some tests gave little or no color whatever. No explanation can be offered for this phenomenon. The final alkalinity was shown not to be a factor. In the procedure reported below, sodium hydroxide was used as the base. The reaction was re-examined with respect to temperature, rate of color development, and the final concentrations of sodium hydroxide, blue tetrazolium, and water. A rapid and simple method was evolved which has given excellent replication, is consistent from day to day, and gives linear Beer-Lambert plots.

MATERIAL AND METHODS

Absolute Alcohol.—The absolute alcohol† is redistilled from alkaline blue tetrazolium according to Weichselbaum and Margraf. Redistilling the ethanol twice with blue tetrazolium decreases the blank reading by 0.030 optical density units in comparison to the blank reading for ethanol distilled only once. Twice redistilled ethanol was used throughout.

Blue Tetrazolium.—(3,3¹-dianisole-bis-4,4¹ [3,5 diphenyl] tetrazolium chloride). Blue tetrazolium is recrystallized from ether according to Weichselbaum and Margraf.

From the Department of Physiology, Western Reserve University School of Medicine, Cleveland, Ohio.

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*Senior Instructor, Department of Physiology, Western Reserve University School of Medicine.

**Technician, Department of Physiology, Western Reserve University School of Medicine.

†Rossville Gold Shield, Commercial Solvents Corporation.

Sodium Hydroxide.—Fisher Scientific Company, electrolytic pellets.

Reagents.—Blue tetrazolium solution. The reagent contains 0.25 mg. of recrystallized blue tetrazolium per milliliter of twice redistilled ethanol.

Three-hundredth normal *sodium hydroxide* (freshly prepared for each series of determinations): A 6.0 N aqueous solution of sodium hydroxide is stored in a polyethylene bottle at room temperature. The 0.03 N NaOH solution in ethanol is prepared by appropriate dilution of the 6.0 N aqueous NaOH with ethanol within one hour prior to addition to the colorimeter tubes. It may be noted that the 0.03 N ethanolic NaOH solution prepared as described has a final concentration of water equal to 0.278 M, and that there is no other source of water in the reaction.

As a result of the studies of the various parameters of the reaction recorded in the section on Results and Discussion, a final procedure was evolved which consists of the following:

Final Procedure.—Known amounts of pure steroids in methanol, or unknown amounts of steroids in washed chloroform extracts of enzyme reactions[5] are added to 13 × 100 mm. culture tubes which have been selected for size and calibrated to serve as colorimeter tubes. The solvent is evaporated under a gentle stream of air which has been passed through a column of activated charcoal and absorbent cotton. To the dried-down residue in the colorimeter tubes is added 2.0 ml. of the blue tetrazolium solution, followed by 1.0 ml. of the 0.03 N NaOH solution. The color is allowed to develop at room temperature with no precautions observed to protect the tubes from either ordinary diffuse daylight or fluorescent lighting. The time intervals required for maximum color development at room temperature depend on the particular steroid used. Table I lists four steroids tested and the times required for maximum color development. The tubes are read at 525 mμ in a Coleman Junior spectrophotometer, Model 6A, adapted to hold the colorimeter tubes described. The quantity of color decreases very slowly on standing (see below); hence colorimeter readings are taken at the prescribed time for maximum accuracy.

TABLE I. OPTIMUM INCUBATION TIME FOR VARIOUS STEROIDS

STEROID	MINUTES
Cortisone	30
Desoxycorticosterone	30
Corticosterone	50
Hydrocortisone	50

Conditions: as in final procedure.

RESULTS AND DISCUSSION

Concentration of Blue Tetrazolium.—Data on the effect of varying concentrations of blue tetrazolium on the final color development are given in Fig. 1. On the basis of the data of Fig. 1, a final concentration of 500 mcg. of blue tetrazolium was used per color reaction. It may be noted that the conditions used for the data of Fig. 1 were those of Weichselbaum and Margraf. These conditions were gradually changed as the study progressed. The fact that linear Beer-Lambert plots are obtained with our final conditions (see below) indicates that the concentration of blue tetrazolium selected is sufficient over the range of steroid concentrations analyzed (9 to 36 mcg. per tube). With the assumption of a molecular weight of 350, the final concentration of the tetrazolium at 500 mcg. per 3.0 ml. is 4.76×10^{-4}M. The final concentration of blue tetrazolium is approximately 10 to 60 times the final steroid concentration on a molecular basis.

Concentration of NaOH.—Ethanolic NaOH was used as the color developing alkali reagent because of unreliability of various tetramethyl ammonium hydroxide solutions, as well as the ready availability of sodium hydroxide of the

TABLE II. EFFECT OF NaOH CONCENTRATION ON BT REACTION

FINAL NORMALITY OF NaOH	OPTICAL DENSITY AT 510 mμ*
1×10^{-5}	Zero
1×10^{-4}	Zero
1×10^{-3}	0.538
1×10^{-2}	0.660
3.33×10^{-2}	0.660

Conditions, per colorimeter tube: 18 mcg. cortisone, 2.0 ml. of ethanol (100 per cent, twice redistilled) containing 500 mcg. blue tetrazolium, 1.0 ethanolic NaOH of various concentrations, 50 minutes' incubation at 37° C., 3.0 ml. final volume.

*Read against ethanol as blank.

highest purity. We have not tested choline, which was shown by Chen and co-workers[1] to yield a more sensitive reaction than NaOH. However, our method with NaOH is equal in sensitivity to that obtained by Chen through use of choline. The choice of the final concentration of sodium hydroxide was based on the data of Table II. It may be noted that full color development is reached at a final concentration of 0.01 N NaOH. This experiment was conducted on three occasions with the same results.

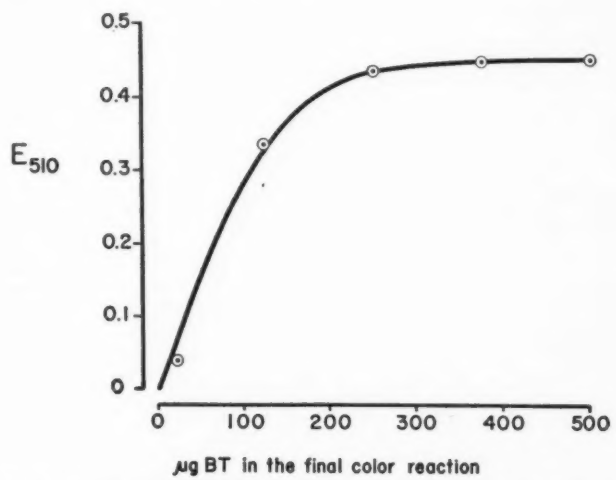

Fig. 1.—Blue tetrazolium concentration. Conditions per colorimeter tube: 20 mcg. DOC, 2.0 ml. of 90 per cent ethanol containing blue tetrazolium, 1.0 ml. of 0.1 N tetramethyl ammonium hydroxide (1 N aqueous tetramethyl ammonium hydroxide diluted 1/10 V/V with 90 per cent ethanol). The tubes were preincubated 20 minutes at 37° C. before addition of tetramethyl ammonium hydroxide. Color was allowed to develop at 37° C. for an additional 20 minutes. Optical density was read at 510 mμ in the Coleman Junior spectrophotometer, against a blank which contained all the reagents except DOC.

Effect of Water.—The presence of water has been found to inhibit color development (Fig. 2). The inhibitory effect of water dictated the use of 100 per cent ethanol in the preparation of the reagents. With reagents prepared as described, the final water content in the reaction is 0.167 per cent or 0.093 M.

Rate of Color Development.—Data of Fig. 3 show the typical time course of the reaction at room temperature. It may be noted that there is an initial rapid color development followed by a slow phase. The slow phase of color development parallels the slow color development in the blank. The kinetics of the reaction imply that there is an initial rapid reduction of the blue tetrazolium

by the steroid, accompanied by a further *slight* reduction of the dye by oxidation products of the ethanol. However, the oxidation of ethanol does not occur to any appreciable extent in the absence of blue tetrazolium. This was indicated by an experiment in which the 0.03 N ethanolic NaOH was allowed to stand for 10, 20, 30, and 40 min. at room temperature before addition to the blue tetrazolium in ethanol. The extent of color developed in five minutes of further incubation was constant regardless of the age of the ethanolic NaOH solution.

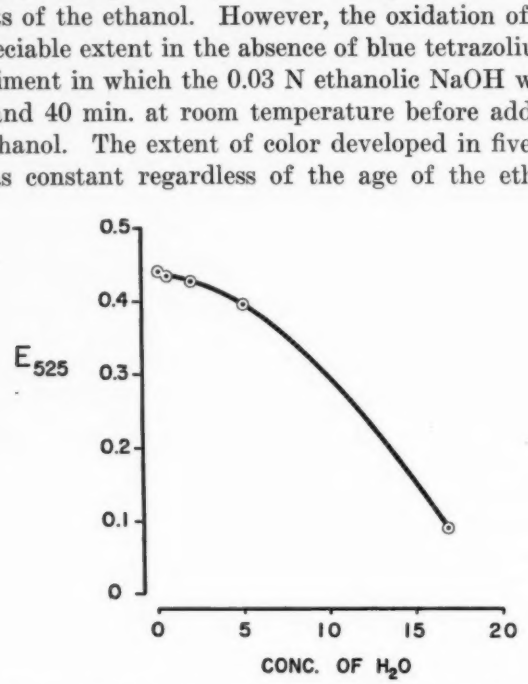

Fig. 2.—Effect of water on reduction of blue tetrazolium. Conditions, as given in Final Procedure, 18 mcg. cortisone per reaction. The abscissa records final concentration of water in per cent by volume.

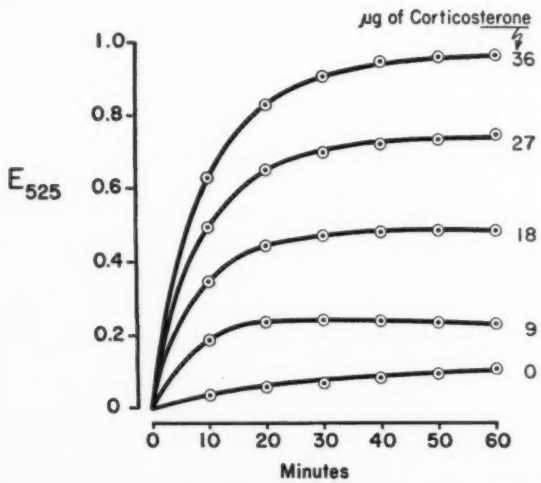

Fig. 3.—Time course of blue tetrazolium reaction. Conditions, as in Final Procedure. Figures denote micrograms corticosterone present per colorimeter tube.

In other experiments the ethanolic NaOH was prepared at least one hour before use with similar results. It may be noted that with small amounts of steroid (Fig. 3, 9 mcg.) there is a slow fading of the color on standing. For maximum accuracy the time for color development is selected so that full color develop-

ment is reached in those tubes containing larger amounts of steroid before the color fades in the tubes containing less steroid. This range is fairly broad. The optimum incubation periods are given in Table I.

Effect of Temperature.—Other investigators have used room temperature,[2, 4] 25° C.,[1] 37° C.,[3] and 90° C.,[4] for reduction of blue tetrazolium. Our results indicate that there is no advantage in conducting the reaction at temperatures other than room temperature. The data of Table III indicate that after 20 minutes the final color development, corrected for the corresponding blank values, is the same at 20° C. and at 37° C., and somewhat low at 60° C. This implies that the secondary reaction due to oxidation of the ethanol in the presence of blue tetrazolium occurs to the same extent in the tubes containing steroid as it does in the blank, at least at lower temperatures. However, it may be noted (Table III, blank [20 min.]) that the color developed in the blank is markedly dependent on the temperature and increases rapidly as the temperature is raised. The low color in the blank at 20° C., and the fact that final color development when corrected for the corresponding blank values was the same over the range 20° C. to 37° C., indicates that over the range indicated no special precautions are necessary regarding the temperature of the reaction.

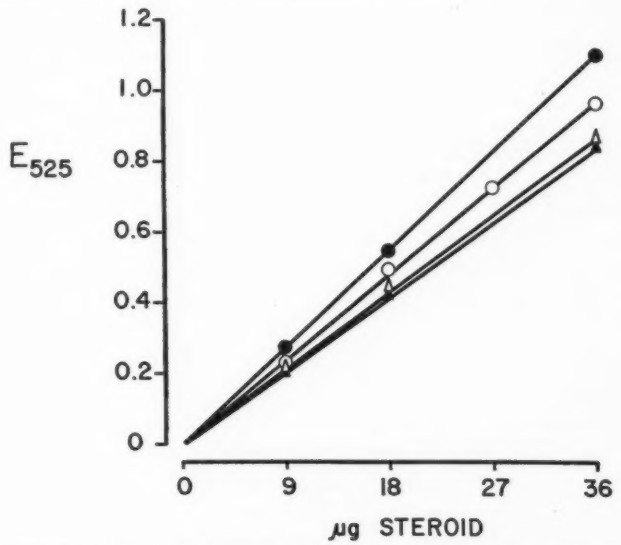

Fig. 4.—Beer-Lambert curves for four adrenocortical steroids, blue tetrazolium reaction. Conditions, as in Final Procedure. Desoxycorticosterone, ●—●; corticosterone, ○—○; cortisone, △—△; hydrocortisone, ▲—▲.

Photosensitivity.—Other authors have emphasized guarding against photosensitivity of blue tetrazolium. Under our conditions no evidence was found to warrant such precautions. A solution of blue tetrazolium in ethanol (0.25 mg. per milliliter) was prepared and allowed to stand in a 50 ml. graduated cylinder exposed to fluorescent light. Color development with cortisone was carried out as described in the section of Final Procedure. The data of Table IV indicate that no destruction of blue tetrazolium occurred even after 22 hours. Reproducibility of duplicates may be noted.

With use of the conditions described, standard curves for four adrenocortical steroids were constructed (Fig. 4). The strict adherence to the Beer-Lambert law is evident.

TABLE III. FINAL COLOR DEVELOPMENT AT VARIOUS TEMPERATURES

MINUTES OF COLOR DEVELOPMENT	OPTICAL DENSITY AT 510 mμ*		
	20° C.	37° C.	60° C.
5	0.323	0.386	0.337
10	0.374	0.393	0.333
20	0.394	0.389	0.309
30	0.393	0.394	
40	0.389	0.388	
50	0.389		
Blank (20 min.)	0.067	0.120	0.521

Conditions: as in Final Procedure, 18 mcg. cortisone per tube.
*Corrected for corresponding blank readings.

TABLE IV. STABILITY OF ETHANOLIC SOLUTION OF BLUE TETRAZOLIUM

HOURS UNDER FLUORESCENT LIGHT	OPTICAL DENSITY
0	0.236, 0.230 (duplicate values)
3	0.230, 0.220 (duplicate values)
5	0.220
22	0.230

Conditions: as in Final Procedure, 9 mcg. cortisone.

SUMMARY

A rapid and simplified method has been presented for the quantitative analysis of adrenocortical steroids by reduction of blue tetrazolium.

ADDENDUM

This method was checked (courtesy of Dr. Robert C. Haynes, Department of Pharmacology, Western Reserve University) and found to be satisfactory over the range of 10 to 50 mcg. of cortisone. The conditions were as described. The reagents used were: Gold Shield absolute ethanol, not redistilled; blue tetrazolium (Nutritional Biochemicals Corp.), used without recrystallization; and sodium hydroxide (Merck), analytical reagent grade.

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VIABILITY OF ERYTHROCYTES OF ACID CITRATE DEXTROSE BLOOD COLLECTED AND STORED IN AND INFUSED FROM AGED PLASTIC BAGS

ALEXANDER P. REMENCHIK, M.D., JOSEPHINE M. DYNIEWICZ, PH.G., JAMES A. SCHOENBERGER, M.D., AND ARNOLD E. OSTERBERG, PH.D.
CHICAGO, ILL.

WITH THE TECHNICAL ASSISTANCE OF D. J. CAMPBELL AND P. HELGREN

INTRODUCTION

A RECENT report[1] presented data collected during a study of the factors affecting the viability of erythrocytes collected and stored in, and infused from, plastic bags containing acid citrate dextrose (ACD) solution. Aged bags containing ACD solution were not investigated in that study; therefore, a study of aged plastic bags* was done, and the results are reported in this paper.

METHODS

Laboratory Techniques.—Red cell survival time was determined by the method of Gibson and Scheitlin[2] with the following minor modifications: (a) 3 ml. samples of whole blood, hemolyzed by saponin, were used for assaying of radioactivity; (b) the last sample drawn on the day of injection is our reference sample; (c) the hematocrit of each sample was corrected to the hematocrit of the reference sample; (d) the samples were assayed for radioactivity at irregular intervals during the course of the study, and the activity of any specific sample was compared to the activity of the reference sample counted at the same time. This per cent survival was then corrected for immediate hemolysis by multiplying the per cent survival by the quantity $\frac{\text{reference sample}}{\text{100 per cent retention value}}$. Osmotic fragility of the red blood cells was determined by measuring the percentage of hemolysis in 0.6 per cent NaCl solution, complete hemolysis in distilled water being used as reference.[1] Plasma inorganic phosphates were determined by the method of Fiske and Subbarow.[3]

Short Exposure Study.—Four Pliapak blood bags, serial number 659-3611, with a shelf life of 180 days, were used in this study. Approximately 500 ml. of blood was drawn into a bag from a clinically healthy adult. About one hour later the cells were tagged with $Na_2Cr^{51}O_4$.* The blood was then reinfused rapidly into an antecubital vein of the donor from whom the blood had been obtained and blood samples were drawn for radioactive assay from the antecubital vein of the opposite arm. Fifty milliliters of blood was retained in the bag and the bag stored for 21 days at 5° C. for the osmotic fragility and inorganic phosphorus studies.

Long Exposure Study.—Two Pliapak bags, serial number 654-8904, with a shelf life of 15 months, were used in this study. The procedure was as described in the short exposure

From the University of Illinois Department of Medicine, College of Medicine, Chicago, Ill., and Abbott Laboratories, North Chicago, Ill.

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study, except that the blood-containing bags were stored for 21 days at 5° C. prior to reinfusion. Only the 24-hour Cr^{51}-tagged red cell survival was obtained in this study.

RESULTS AND DISCUSSION

Survival.—The data for immediate survival, radioactive half life, life-span index, and 24-hour survival are presented in Table I. An immediate survival of 70 per cent or more after 21 days of storage, and a life-span index 1.80 or less are considered acceptable.[1] The 94 per cent immediate survival in Patient Grace B. may be an artifact due to difficulty in maintaining reinfusion of blood.

Phosphates.—Table I gives the plasma inorganic phosphorus levels. Upper limits of normal are considered to be 10 to 15 mg. per 100 ml. plasma, after 21 days of storage.[1]

Osmotic Fragility.—Table I gives the red cell osmotic fragility in 0.6 per cent NaCl solution. Upper limits of normal for blood stored for 21 days are 10 to 15 per cent hemolysis.[1]

TABLE I. Cr^{51} ERYTHROCYTE SURVIVAL DATA, PLASMA INORGANIC PHOSPHORUS, AND OSMOTIC FRAGILITY OF BLOOD COLLECTED AND STORED IN PLASTIC BAGS

DONOR	BAG	COLLECTED	REINFUSED	IMMEDIATE SURVIVAL (%)	24-HOUR SURVIVAL (%)	RADIOACTIVE HALF-LIFE (DAYS)	LIFE-SPAN INDEX	INORGANIC PHOSPHORUS MG./100 ML. PLASMA†	RED CELL FRAGILITY IN 0.6% Nacl % HEMOLYSIS†
Gloria B.	659-3611	8/ 8/55	8/ 8/55	100		35	1.43	4.65	1.06
A. P.	659-3611	8/15/55	8/15/55	100		29	1.72	6.94	2.8
A. L.	659-3611	8/22/55	8/22/55	100		32	1.56	4.77	7.57
Grace B.	659-3611	8/29/55	8/29/55	94		33	1.51	6.58	3.3
R. M.	654-8904	11/18/55	12/ 9/55		86.6			2.09	6.93
M. C.*	654-8904	11/18/55						6.23	5.57

*Refused to continue study after blood drawn.
†After 21 days of storage at 5° C.

SUMMARY AND CONCLUSIONS

Our results suggest that prolonged storage of plastic bags of this type containing ACD solution does not affect the following indices of viability of erythrocytes collected and stored in and infused from plastic containers: (1) Cr^{51}-tagged red cell survival time; (2) immediate postinfusion survival; (3) plasma inorganic phosphorus; and (4) osmotic fragility in 0.6 per cent NaCl solution.

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A PLASTIC COIL SIMPLIFYING RADIOACTIVE LIQUID PHASE COUNTING

SOLOMON N. ALBERT, M.D., WM. A. SPENCER, M.D., MURRAY FINKELSTEIN, PH.D., JO SHIBUYA, M.D., SEYMOUR ALPERT, M.D., AND CHARLES S. COAKLEY, M.D.
WASHINGTON, D. C.

SEVERAL techniques[1-12] are available today for blood volume determination, most of which involve intravenous administration of radioisotopes and radioactive liquid phase counting. The present study has been an endeavor to simplify the technical procedures involved in the radioisotope methods. A plastic coil was devised and fixed in a well-type scintillation counter and sample radioactive fluids were drawn into or passed through this coil for radioactivity analysis. The results obtained in this manner, in both in vitro and patient studies, were compared statistically with those obtained utilizing the standard method of counting radioactivity in test tube aliquots of fluids. There was no essential difference between them. The coil method has proved efficient and has been used for serial blood volume studies, and with an arteriovenous shunt through the coil, in continuous blood volume estimation and hemodynamic studies.

METHOD

Polyethylene tubing with an internal diameter of 1.77 mm. (Clay-Adams No. PE260) was wound around a 5 mm. glass tube forming a two-layered coil which fit into a standard Nuclear Chicago well-type scintillation counter (Model DS-3) (Fig. 1). The two leads from the coil were taped at the point of emergence from the well-cap and also to the external lead shield. One end of the coil tubing was connected to a 30 ml. syringe and the other was used for aspirating samples from a container or directly from a patient's circulation; or both ends were used in an arteriovenous shunt.

Washed human erythrocytes tagged with Cr^{51} according to the method of Gray and Sterling[6] and saline solution of radioactive chromium as $Na_2Cr^{51}O_4$ were prepared in final concentrations of 1 μc per 1.2 to 2.0 ml. of suspended cells or solution. Aliquots of these were used as standards. In addition, standards of 1 ml. aliquots, measured in 1 ml. pipettes diluted to 100 ml. in volumetric flasks, were also employed. These were drawn into the coil and counted. Thirty milliliters of undiluted cell suspension or Cr^{51}–saline solution, measured in a syringe, containing a total of approximately 15 to 25 μc, was then added to a measured volume of saline in a glass jar. Patients received only washed-cell suspensions in doses of 30 ml. Following adequate mixing (10 to 30 minutes in patients) samples were immediately drawn into the coil and counted. In one series of experiments, determinations were repeated with use of the standard test-tube method of liquid phase counting. Two milliliter aliquots of each standard and dilution were saved in test tubes and counted in the same well-type scintillation counter after completion of an experiment and removal of the coil. The volumes estimated by the two methods were then compared statistically with each other and also with the measured total fluid volume.

From the Departments of Anesthesiology, District of Columbia General Hospital and the George Washington University Hospital, Washington, D. C.
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All counts were for two minutes preset time, counted at least in duplicate, and usually duplicate samples were analyzed for radioactivity.

After the counting of each sample, the coil was flushed with 5 to 10 ml. of saline or up to 20 ml. when undiluted cell suspension or whole blood was being counted. Background count consisted of an average value taken during a period of at least 30 minutes immediately prior to sample counting and spot checked between samples as well as upon completion of an experimental run.

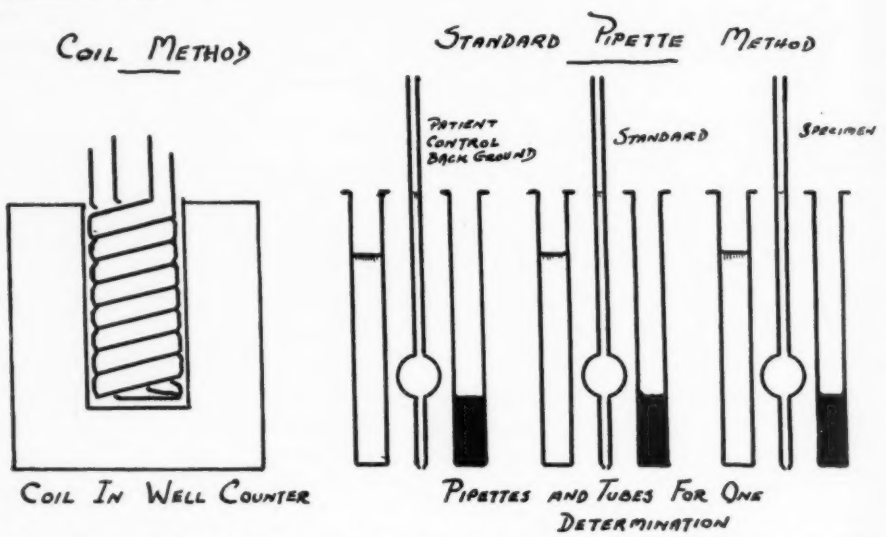

Fig. 1.—The plastic coil in well counter; and comparison of glassware employed in the coil and standard test tube methods.

Total fluid volume was calculated as follows:

$$1.\ \frac{\text{Counts of standard} \times 30}{\text{Counts of sample—background}} = \text{total volume}$$

When the 1:100 diluted standard was employed, the result was multiplied by a factor of 100.

The reliability of the method was tested by a second and third determination on the initial volume with additional 30 ml. volumes of standard Cr^{51}–saline solution or –tagged cells. Total fluid volumes were then calculated by the following two methods: (a) using Formula 1 but considering the total volume of added standard as the multiplicand in the numerator; and (b) using the following formula:

$$2.\ \frac{\text{Counts of standard} \times 30}{\text{Counts of sample—control background}} = \text{total volume}$$

"Control background" is the activity prior to addition of a successive 30 ml. volume of radioactive cell suspension or solution.

To further test the method, we removed a measured amount of the radioactive fluid from the jar and replaced with nonactive saline. Formula 2 was then employed for calculation of the total fluid volume.

RESULTS

The results of a series of five in vitro experiments are summarized in Table I. In this series, the volumes were estimated by both the coil and test-tube methods. Furthermore, a standard diluted 1:100 was employed simultaneously with an undiluted standard in all volume determinations. There was no statistically significant difference in the coil technique between: (a) measured and estimated

volumes ($p = >0.8$ to <0.5); (b) serially estimated volumes using both Formulas 1 and 2 ($p = 0.8$ to >0.3); (c) estimated volumes obtained by Formula 1 when compared with those obtained by Formula 2 ($p = >0.7$ to >0.6); or (d) volumes estimated with dilute and undiluted standard ($p = >0.9$ to <0.7). The mean difference in estimated volumes between the two methods employed was in a range of –3.31 to +2.59 per cent, with use of both formulas for the calculations. The difference between estimated volumes before and after removing 500 ml. of active solution from the gross volume and replacing it with nonactive saline was in a range of –2.5 to +7.8 per cent with a mean difference of 0.5 ± 1.9 per cent.

TABLE I. ESTIMATED VOLUMES WITH Cr^{51}–TAGGED ERYTHROCYTES. AVERAGE RESULTS OF FIVE IN VITRO EXPERIMENTS

VOLUME NO.	MEASURED VOLUME (ML.)†	(1)‡ ESTIMATED MEAN VOLUME (ML.)	(1)‡ RANGE OF DIFFERENCE (PER CENT)	(2)§ ESTIMATED MEAN VOLUME (ML.)	(2)§ RANGE OF DIFFERENCE (PER CENT)
		A. Coil Method—Standard Undiluted			
1	3,530	3,501 ± 40	–4.8 to +1.1	- - - - -	- - - - -
2	3,550	3,546 ± 38	–3.2 to +2.2	3,595 ± 86	–4.6 to +8.5
3	3,570	3,549 ± 27	–2.7 to +1.1	3,560 ± 16	–1.7 to +0.5
4*	3,590	- - - - -	- - - - -	3,576 ± 56	–3.3 to +5.4
		B. Coil Method—Standard Diluted 1:100			
1	3,530	3,510 ± 39	–4.3 to +2.3	- - - - -	- - - - -
2	3,550	3,553 ± 36	–2.7 to +2.1	3,604 ± 85	–3.5 to +9.2
3	3,570	3,558 ± 25	–2.4 to +1.2	3,569 ± 12	–1.1 to +0.5
4*	3,590	- - - - -	- - - - -	3,585 ± 60	–3.5 to +6.0
		C. Test-Tube Method—Standard Undiluted			
1	3,530	3,385 ± 19	–5.5 to –2.4	- - - - -	- - - - -
2	3,550	3,448 ± 24	–5.2 to –1.1	3,517 ± 66	–6.0 to +4.4
3	3,570	3,446 ± 36	–6.1 to –0.3	3,454 ± 74	–8.3 to +2.7
4*	3,590	- - - - -	- - - - -	3,580 ± 55	–4.3 to +3.5
		D. Test-tube Method—Standard Diluted 1:100			
1	3,530	3,478 ± 25	–2.8 to +0.2	- - - - -	- - - - -
2	3,550	3,506 ± 29	–3.0 to +1.8	3,615 ± 81	–3.9 to +7.4
3	3,570	3,543 ± 42	–4.0 to +2.6	3,530 ± 60	–4.8 to +3.1
4*	3,590	- - - - -	- - - - -	3,678 ± 57	–2.1 to +6.5

*Five hundred milliliters of active saline was removed from the gross volume and replaced with 500 ml. of nonactive saline.

†Thirty milliliters of tagged cells was added for the volume estimation after removal of 10 ml. aliquots for test-tube counting. Therefore increments of only 20 ml. in total measured volumes.

‡Formula 1 employed for volume estimations.

§Formula 2 employed for volume estimations.

Consequently, in the subsequent series of ten experiments it was deemed unnecessary to continue the comparison of diluted and undiluted standard as well as the coil and test-tube methods. Therefore, only the coil technique was employed and the standard was undiluted. The measured initial volumes were between 400 and 5,000 ml. of saline. In this series, both Cr^{51}–tagged cells and Cr^{51} as sodium chromate in saline solution were employed, frequently in the same experiment. Proportionately lesser quantities of these were used for estimation of the smaller volumes, i.e., approximately 2.5 to 4 μc in 5 ml. Up to ten determinations were performed in each experiment. This included repeated determinations with or without gross changes in the total volumes. The errors in the results obtained were usually less than 4 per cent and always within the limits seen in Table I.

This method for determining volume was also applied to a series of patients. In some of these the two techniques were compared, as well as determinations with diluted and undiluted standard. Since the difference between results obtained by the two methods was comparatively negligible, less than 3 per cent, and the difference between estimated volumes based on diluted and undiluted standard was less than 2 per cent, this comparison was discontinued. As in the previous in vitro experiments, the reliability of the method was tested by total blood volume determinations after successive doses of tagged cells and also after blood donations. The estimated volume difference was never greater than 8.5 per cent after any successive determination, usually less than 5 per cent.

DISCUSSION

Utilizing the above described plastic coil in a well-type scintilation counter, we have been able to determine fluid volumes with greater speed and with accuracy and reproducibility comparable to that achieved by the test-tube method. Geometry and volume are not altered during an entire procedure, therefore eliminating the need for calibrating glassware (see Fig. 1 for comparison of glassware employed in both techniques) and for excessive caution in measuring liquid samples. The possibility of technical errors is thereby diminished. Furthermore, the accuracy of estimated volumes was independent of the concentration of the standard employed. This, therefore, allows direct administration of tagged cells to a patient through the coil in a closed system. Thus, serial blood volumes, or with an arteriovenous shunt through the coil, continuous total volumes and hemodynamic changes may be determined. These results also suggest the coil technique as a general procedure for liquid sample counting.

The polyethylene coil showed no retention of radioactivity from Cr^{51}-tagged red cell suspensions when it was washed with relatively small amounts of saline. However, there was evidence of radioactivity adherence when aqeous solutions of radioactive sodium chromate were used in concentrations above 1 μc per milliliter. Similarly, saline solutions of radioactive iodinated human serum albumin and aqueous solutions of *free* iodine (NaI^{131}) showed a tendency to adhere to the coil. A measure of decontamination from the serum albumin was achieved by several washings with laboratory detergent. However, radioactive iodinated serum albumin diluted in plasma or whole blood presented no problem in concentrations up to 0.2 μc per milliliter and was readily washed out with normal saline. Higher concentrations were not tested.

Variations in the temperature of the chromate solution between 10° and 40° C. did not show any appreciable difference in count. There was only a one per cent variation in counts between either extremes of temperature, and no change within 20° to 40° C.

The coil technique has been applied in serial and continuous blood volume determinations in a series of 63 patients to date. These determinations covered preoperative blood volume levels and serial determinations during surgery as well as during the immediate postoperative period. Similarly, blood volumes were determined on patients scheduled for minor elective operations, and the reliability of the method was tested, as in the in vitro studies, by a second and

third administration of the tagged cells and also after blood donations. The coil has also been employed satisfactorily in an arteriovenous shunt. These investigations are continuing and will be the subject of a later report.

Preliminary in vitro experiments supported the predicted increased accuracy and reproducibility of results with doses of approximately 20 μc of $Cr.^{51}$. It is also desirable to maintain the patient's radioactivity level at several times atmospheric background for serial determinations, particularly during surgery, thus decreasing the percentage error in volume following subsequent doses of tagged cells.

Investigations are now underway utilizing doses of 3 to 4 μc of radioactive human serum albumin in these studies.

SUMMARY

1. A simplified method for radioactive liquid phase counting has been developed utilizing a plastic coil. This has been applied specifically to volume determinations.

2. In vitro experiments showed that estimated volumes did not differ significantly from measured volumes and were comparable to the results obtained by the test-tube method of liquid phase counting.

3. Rapid reproducible results have also been obtained in patient studies.

4. By the production of an arteriovenous shunt through the coil, continuous blood volume estimations, determinations of cardiac output, circulation time, and circulatory hemodynamic changes can be studied.

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A STUDY OF THE DENSITOMETRIC METHOD OF ANALYZING FILTER PAPER

Electrophoretic Patterns of Serum

Gerald Klatskin, M.D., Oscar M. Reinmuth, M.D.,* and Waddell Barnes, M.D.**
New Haven, Conn.

OF THE several methods available for estimating the composition of the serum proteins, those based on electrophoresis appear to provide the most useful and reliable information. Unfortunately, even the simplest of these, the filter paper method, is still too time consuming to permit its adoption as a routine procedure. For that reason attempts have been made to further simplify the technique by scanning the stained patterns in a densitometer[1-10] and by recording and integrating their density curves automatically,[11, 12] thus eliminating the necessity for elution, colorimetry, graphing, and planimetry, the steps in the usual procedure that are most laborious.

Most investigators[1, 8, 9, 12] have found that the scanned density of stained protein on paper does not bear a linear relationship to concentration, the ratio between the two tending to fall off as the concentration rises. Nevertheless, many[1, 8, 12] consider direct scanning a valid method of analyzing serum electrophoretic patterns, since it has been shown that the densitometer response is essentially linear at the low concentrations normally found in the globulin fractions, and that at the higher concentration of albumin, a greater dye-binding capacity tends to compensate for deviations from Beer's law. As supporting evidence these authors have reported reasonably good agreement between the results of direct densitometry and moving boundary electrophoresis. However, others[7, 10] have found a marked discrepancy between the two, which they attribute to scanning errors.

The deviations from Beer's law encountered in direct densitometry have been variously ascribed, wholly or in part, to (a) light scatter,[9] (b) variations in the dye-binding capacity of protein dependent on its concentration and area of distribution,[13, 14] (c) "edge effects" attributable to the tendency of highly concentrated protein to spread beyond the limits of the scanning slit,[6] (d) failure to use monochromatic light,[6, 12] and (e) irregular transmission of light through the filter paper pattern due to uneven illumination of the densitometer

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn. This study was supported by an Institutional Grant from the American Cancer Society.

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*Formerly trainee of the National Institute of Arthritis and Metabolic Diseases and Clinical Fellow in Medicine. Present address: Harvard Neurological Unit, Boston City Hospital, Boston, Mass.

**Formerly A. B. Coxe Research Fellow in Medicine. Present address: 781 Spring Street, Macon, Ga.

slit,[6] uneven application of the protein streak[1] or irregular distribution of protein related to the fibrillar structure of paper.[9, 12] The present investigation was undertaken to define the relative importance of these factors in producing scanning errors, to test the validity of the scanning method in the analysis of serum, and to evaluate the performance of a recently developed recording densitometer* designed to compensate for scanning errors.

METHODS

Filter Paper Electrophoresis.—The procedure was carried out on strips of filter paper measuring 3 × 30 cm. in the hanging-strip chamber described by Williams, Pickles, and Durrum.[15] Whatman No. 1 paper was used in all experiments except those with the Analytrol, in which case Whatman 3 MM paper was used. The technique employed was essentially that of Jencks, Jetton, and Durrum[12] and entailed the use of (a) Veronal buffer, pH 8.6, ionic strength 0.075, (b) a constant current of 7.5 Ma. for 8 strips for 16 hours at room temperature, (c) oven-drying at 110° C. for 30 minutes, (d) staining in 0.01 per cent bromphenol blue† in 5 per cent acetic acid and 5 per cent zinc sulphate for 16 hours, (e) two ten-minute rinses in 5 per cent acetic acid, (f) fixation for ten minutes in 0.3 per cent sodium acetate in 5 per cent acetic acid, and (g) blotting followed by drying at 110° C. for 20 minutes.

Except where otherwise indicated, the volume of protein applied was 0.01 ml. In experiments involving the comparison of varying concentrations of protein the same calibrated transfer pipette was used throughout to minimize errors in delivery. In analyzing serum, the measured volume of protein was transferred to a mechanical applicator‡ which was then applied to the paper. This assured a more even distribution of protein than was possible by streaking directly with a pipette. Preliminary recovery experiments revealed that delivery was almost the equal of pipetting in accuracy.

Densitometry.—The instrument used routinely, a modification of the Model 525 Photovolt electronic densitometer,§ had a pinpoint incandescent light source and cylindrical lens, a phototube with a 4 cm. cathode, a 25 × 1 mm. slit, and a removable Bausch and Lomb interference filter (transmission maximum 600 mμ, band pass 20 mμ) interposed between the slit and the phototube. The strip, which was held between 1 mm. plates of optical glass in a metal frame, could be advanced by means of a geared track calibrated to stop at 1 mm. intervals. The accuracy of the transport mechanism was checked by scanning a strip of light-sensitive photographic film. This revealed that there was no overlapping or separation of the slit images and that the illumination was relatively uniform. However, it was found by direct measurement that the light was more intense in the center of the slit than at either end.

The densities were plotted against migration in millimeters on a good grade of graph paper, following which the areas under the peaks were excised, weighed on a sensitive balance, and compared on the basis of their weights. This was found to be the equal of planimetry in accuracy and reproducibility and had the advantage of greater speed. In some experiments the "areas" were determined by adding the densities recorded at 1 mm. intervals and were expressed as total density.

Elution.—The stained strips were cut transversely at 5 mm. intervals and eluted into 6 ml. aliquots of 0.01 N NaOH for a period of 30 minutes. An eluate of an unstained segment of identical width served as a blank. Except where otherwise stated, the optical density was determined in a Coleman Junior spectrophotometer at 590 mμ, appropriate dilutions being made to keep the density below 0.650, the upper limit of the linear range of the instrument for bromphenol blue solutions.

*The Analytrol, Specialized Instruments Corporation, Belmont, Calif.
†Bromphenol blue, Hartman-Leddon Company, Philadelphia, Pa.
‡Specialized Instruments Corporation, Belmont, Calif.
§Photovolt Corporation, New York, N. Y.

Moving-Boundary Electrophoresis.—Serum was diluted 1 to 4 with Veronal buffer of pH 8.6 and 0.1 ionic strength and dialyzed with continuous stirring against buffer at 5° C. for 24 hours. The analyses were then carried out in the standard cell of an Aminco-Stern apparatus. An enlargement of the ascending pattern was traced on graph paper and the relative concentrations of the fractions calculated in the usual manner.

Albumin-Soaked Paper.—Strips of Whatman No. 1 paper measuring 3 × 19 cm. were folded 2.5 cm. from one end and tacked to rubber stoppers. The latter were then tightly fitted into 3.8 × 20 cm. tubes containing a 5 cm. layer of albumin solution. Care was taken to keep the strips centered so that they did not adhere to the sides. The tubes were held in the vertical position in a rack and stored at 5° C. for 18 hours. Following removal of the strips, 2 cm. segments were cut off at each end. The remaining segment was suspended horizontally and allowed to dry in the air following which it was oven-dried and stained in the usual manner.

The mean optical density of the strip was determined by scanning a 4 × 1 cm. segment removed from its center with use of a special 6 × 1 mm. slit. The total dye content of the scanned area was then determined by elution.

The Analytrol.—This newly developed densitometer incorporates two highly desirable features, a method for recording transmitted light values in terms of density while correcting for deviations from Beer's law, and a mechanical device for simultaneously integrating the density curves obtained. The scanning slit and photocell are illuminated by a filtered incandescent lamp which also activates a balancing photocell situated behind a slit whose length can be varied by means of a rotating cam. The outputs of both cells, which are equalized mechanically with an unstained segment of paper in position, are fed alternately into an amplifier. As a stained area of the strip is drawn across the light path of the scanning cell, a difference in output results which gives rise to an alternating signal. This is amplified and fed into a motor which rotates the cam until the signals from both photocells are once again equalized. The recording pen, which is linked to the cam, travels a distance proportional to cam rotation and plots its course on a chart moving at right angles. The shape of the cam, which ultimately determines the magnitude of the pen deflection, has been designed on an empirical basis to produce a deflection proportional to the concentration of bromphenol blue-stained protein on Whatman 3 MM filter paper.

RESULTS

Densitometry of Electrophoresed Albumin Streaks.—Electrophoresed albumin* streaks stained with bromphenol blue did not obey Beer's law when scanned in the densitometer. As is evident in Fig. 1, the relationship between total density and concentration was curvilinear, the deviation from linearity increasing with concentration. Neither oiling the paper with a bromonaphthalene-mineral oil mixture[1] nor introducing a 600 mμ light filter compensated for the error.

Amidoschwarz†-stained albumin streaks behaved somewhat differently in that they gave a linear response up to a concentration of 1.5 Gm. per cent on dry paper and 2.5 Gm. per cent on oiled paper (Fig. 2). However, at higher concentrations the same deviation from Beer's law was evident, results which are in accord with those reported by Grassmann and Hannig.[1]

*Salt-poor concentrated human serum albumin obtained from the American Red Cross through the courtesy of Dr. S. T. Gibson.

†Amidoschwarz 10 B, Farbenfabriken Bayer, Leverkusen, Germany.

The tendency for the scanned density to fall off at higher concentrations of albumin did not appear to be related to a decrease in dye-binding capacity, since the amount of dye recovered on elution was proportional to protein concentration over the range investigated (Figs. 1 and 2). It will be noted that with both dyes the elution curve failed to pass through the zero point, an anomaly attributed to incomplete elution or fading.

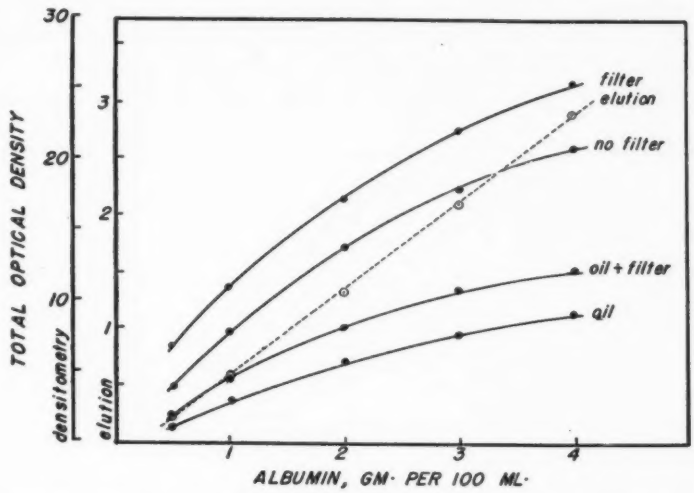

Fig. 1.—Relationship of total scanned density to concentration in albumin streaks electrophoresed on filter paper and stained with bromphenol blue (solid lines), showing the effects of oiling and the introduction of a filter. The amount of dye recovered on elution of the same strips is indicated by the broken line.

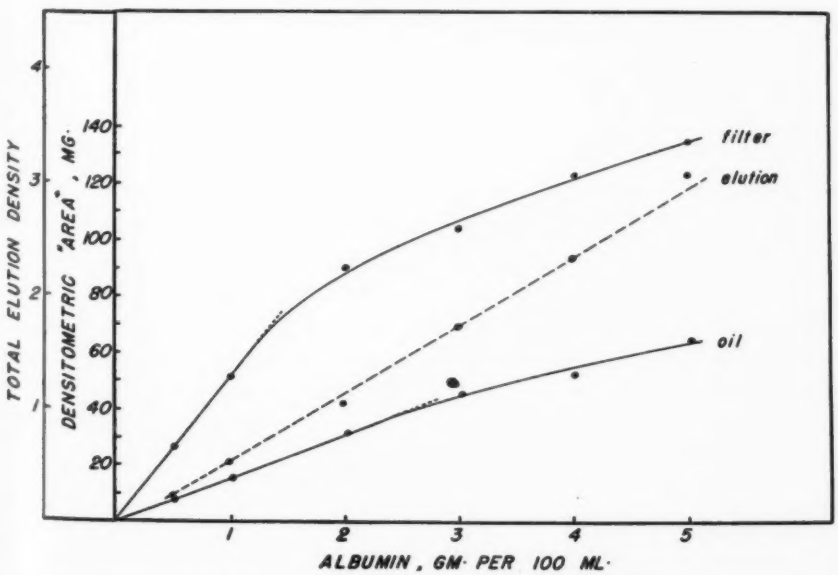

Fig. 2.—Relationship of scanned density, expressed in terms of "area," to concentration in albumin streaks electrophoresed on filter paper and stained with Amidoschwarz 10 B, showing the effects of oiling, and the introduction of a filter (solid lines). The amount of dye recovered on elution of the same strips is indicated by the broken line.

In the preceding experiments not only the peaks but also their trails were scanned and eluted to eliminate errors due to the adsorption of albumin on paper. Omitting the trails from consideration did not alter the curvilinear relationship between scanned density and concentration. However, the amount of dye found in the trail, which increased with the albumin concentration, appeared to be of sufficient magnitude at high concentrations to introduce significant errors in scanning the globulin peaks of serum. Possibly this was an artefact related to the presence of small amounts of globulin in the albumin solution employed, since others[12, 16] have reported that the amount of albumin adsorbed by paper during electrophoresis is constant and independent of concentration.

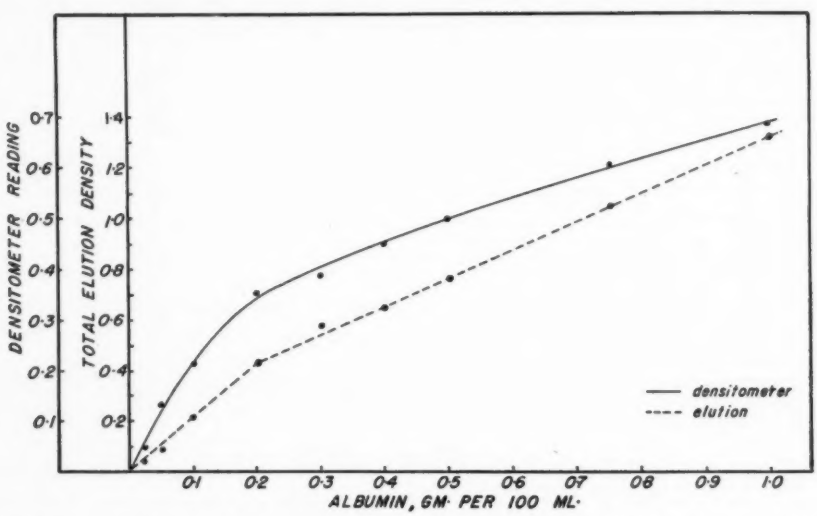

Fig. 3.—Density of bromphenol blue-stained albumin-soaked filter paper measured in a densitometer (solid line) compared with the amount of dye recovered on elution (broken line). The density values plotted represent the means of 40 readings at 1 mm. intervals at each concentration. The elution values represent the amounts of dye recovered from the scanned areas.

The width of electrophoresed protein bands has been found to vary with concentration,[6] an observation that was confirmed by electrophoresing protein streaks of constant length on broad strips of paper. Since the scanning slit of the densitometer was of fixed width, the possibility was considered that the deviation of the density curve from linearity at high concentrations might be related to an increased proportion of dye in the unscanned edges. This proved not to be the case, since the amount of dye eluted from the scanned area alone was proportional to the albumin concentration over the range found in serum. However, at higher concentrations, or as a result of uneven application of the protein streak, "edge effects" became an important source of scanning errors.

Densitometry of Albumin-Soaked Filter Paper.—Since the concentration gradients normally present in electrophoresed protein bands, and those introduced by irregular streaking, were possible sources of scanning errors, the relationship between scanned density and concentration was re-examined in uniformly stained albumin-soaked paper. It was found, as in the preceding ex-

periments, that the densitometer response to increasing concentrations of bromphenol blue-stained albumin was not linear (Fig. 3). However, in this instance the amount of dye recovered on elution was not proportional to concentration, suggesting that a decrease in dye-binding capacity might be responsible for the decline of the density curve at high concentrations.* Nevertheless, when the scanned densities were plotted against the amount of dye eluted instead of the protein concentration, thereby compensating for differences in dye-binding, the deviation from Beer's law was still evident (Fig. 4).

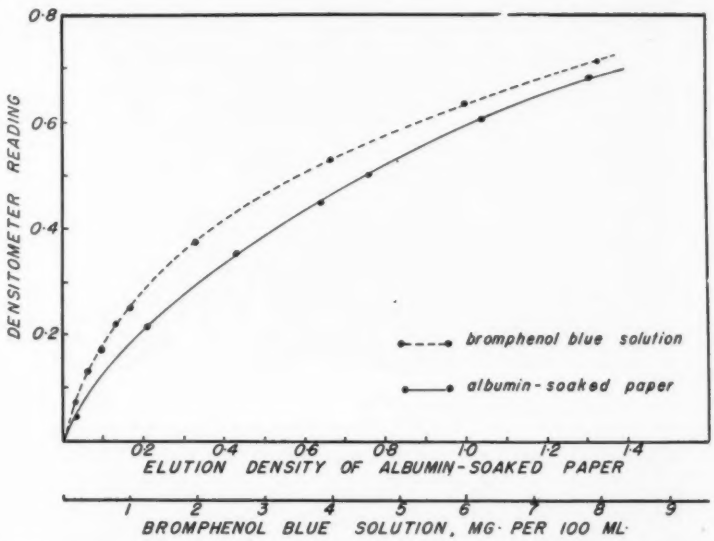

Fig. 4.—Relationship of scanned density to the amount of dye recovered from bromphenol blue-stained albumin-soaked filter paper (solid line). The values plotted were obtained from the data illustrated in Fig. 3. For purposes of comparison the response of the densitometer to solutions of bromphenol blue is shown (broken line).

In view of these observations the failure of electrophoresed albumin streaks to yield a linear densitometer response did not appear to be the consequence of concentration gradients or gross irregularities in streaking. However, microscopic examination revealed that even in uniformly stained albumin-soaked paper the dye was irregularly distributed on the fibers (Fig. 5), a situation inconsistent with a strict linear relationship between density and concentration. According to Beer's law the fraction of light absorbed by a given thickness of solution increases exponentially with increasing concentration of solute, a relationship which is possible only where the light-absorbing molecules are uniformly distributed, so that successive monomolecular layers each absorb the same fraction of light reaching them. Rearrangement of the molecules in an irregular pattern results in a net decrease in density, an effect which becomes more pronounced as the concentration increases. However, when the number

*It is unlikely that the apparent decrease in the dye-binding capacity was due to a decrease in the volume of protein taken up by paper at high concentrations, since the elution curve obtained when known volumes of albumin were applied to dry paper showed the same deviation from linearity. This difference in the behavior of albumin on dry and buffered-moistened paper may account for the failure of some investigators[14] to find a linear relationship between protein concentration and dye uptake.

A.

B.

Fig. 5.—Microscopic view of bromphenol blue-stained albumin-soaked filter paper showing the irregular distribution of dye on paper fibers. *A*, Albumin concentration 1.1 Gm. per 100 ml., density 0.72. *B*, Albumin concentration 0.2 Gm. per 100 ml., density 0.32.

of light-absorbing particles is very large and the irregularities in distribution are randomized, the deviations from Beer's law may be negligible under some conditions. Similarly, while monochromatic light is usually essential for a linear relationship, the deviation with mixed light may be negligible under some conditions. The question arose, therefore, whether the failure of stained albumin streaks to obey Beer's law was due to (a) the irregular distribution of dye on paper fibers, (b) the use of mixed instead of monochromatic light, or (c) an instrument error. An examination of the densitometer response to clear solutions of dye under varying conditions of light gave some indication of the relative importance of these factors.

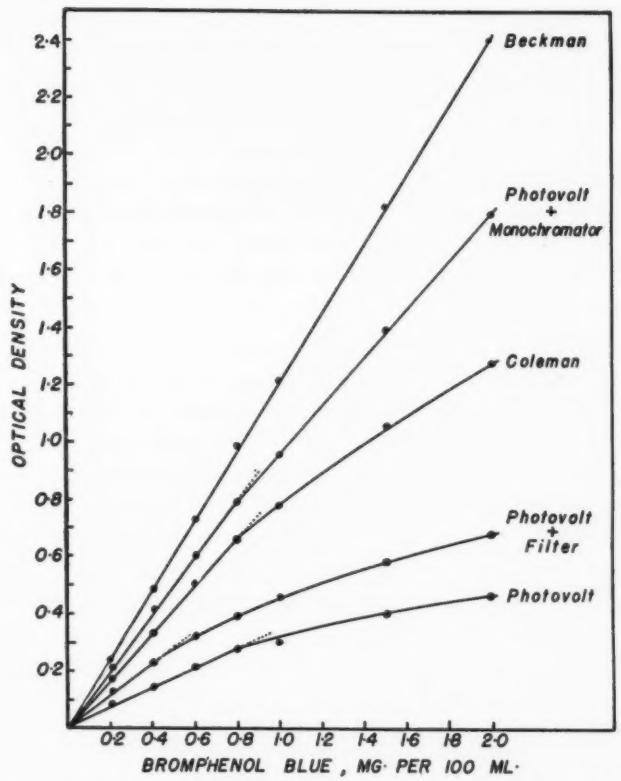

Fig. 6.—Response of the densitometer to increasing concentration of bromphenol blue in solution under varying light conditions compared with that obtained with Beckman and Coleman spectrophotometers set at 590 mμ.

Densitometry of Dye Solutions.—Graded concentrations of bromphenol blue in 2 per cent sodium carbonate in 50 per cent methanol were sealed in 1 cm. Beckman cuvettes and scanned in the densitometer with use of a 1.5 mm. circular aperture. Light leaks were eliminated by masking and by carrying out the procedure in total darkness except for dim galvanometer illumination. With unfiltered incandescent light there was a marked deviation from linearity at a density of 0.27 (Fig. 6). This was not corrected by introducing a 600 mμ

filter. However, when the monochromator of a Beckman spectrophotometer set at 590 mμ was substituted for the incandescent light source,* the response was linear to a density of 0.85, a response inferior to that obtained with the same solutions in a Beckman DU spectrophotometer, but superior to that obtained in a Coleman Junior spectrophotometer (Fig. 6). Solutions of Amidoschwarz examined under the same conditions yielded very similar results.

From a comparison of the density curves obtained with clear dye solutions and stained albumin-soaked paper (Fig. 4), it is evident that the very limited linear range of the densitometer was largely responsible for the deviations from Beer's law encountered when stained protein was scanned (Figs. 1 and 2). No doubt other factors, such as irregularities in dye distribution, light scatter and uneven illumination of the scanning slit contributed to the instrument error, but these appeared to be of secondary importance only.

Since the densitometer was capable of responding linearly over a considerable range when a monochromator served as the light source (Fig. 6), it may be assumed that the deviations observed at relatively low densities under standard operating conditions were related to the use of mixed light, rather than to a defect in the optical or electronic system. Although the latter were less efficient than those in the Beckman spectrophotometer, they were, nevertheless, capable of yielding a linear response with monochromatic light within the range of densities usually found in serum electrophoretic patterns.

Comparison of Densitometers.—The relationship between dye concentration and density was re-examined in three other instruments. One of these, a standard Model 525 Photovolt electronic densitometer, was similar to the one employed in the preceding experiments, except that it had a shorter phototube cathode. The other two, a Model 425 Photovolt densitometer and a Chromaphor I,† resembled the instrument described by Grassmann and Hannig[1] and employed barrier-layer photocells and micro-ammeters without amplification.

In all three instruments, stained albumin-soaked paper, electrophoresed albumin streaks, and clear solutions of dye showed the same deviations from Beer's law previously noted. It is highly probable, therefore, that, in the absence of monochromatic light, most, if not all, densitometers are incapable of yielding a linear response to increasing concentrations of bromphenol blue or Amidoschwarz within the range of densities usually found in serum electrophoretic patterns.

Densitometry of serum.—Ten normal sera were electrophoresed in duplicate on Whatman No. 1 paper and scanned in the densitometer. One strip was streaked with 0.005 ml. of serum,‡ stained with Amidoschwarz, and cleared with a mixture of bromonaphthalene and mineral oil, as recommended by Grassmann and Hannig.[1] The other was streaked with 0.01 ml. of serum, stained with bromphenol blue, and cleared in mineral oil, as described by Jencks, Jetton, and

*This was accomplished by replacing the phototube housing of a Beckman spectrophotometer with a metal plate containing a 1.5 cm. aperture opposite the exit slit. The phototube assembly of the densitometer was then centered over the aperture with masking tape.

†Gamma Instrument Co., Great Neck, N. Y.

‡Grassmann and Hánnig[1] used 0.006 ml. streaked on a 4 cm. wide strip.

TABLE I. COMPARISON OF MOVING BOUNDARY AND FILTER PAPER ELECTROPHORETIC ANALYSES OF 10 NORMAL SERA*

METHOD	ALBUMIN	GLOBULINS			
		α_1	α_2	β	γ
Moving boundary (Tiselius)	57.7 ± 2.7	4.5 ± 1.1	7.2 ± 1.1	15.3 ± 2.4	15.3 ± 1.5
Elution (bromphenol blue)	58.1 ± 4.7	3.4 ± 1.0	8.8 ± 2.0	11.9 ± 2.2	17.8 ± 1.9
Scanning (Amidoschwarz 10B)	49.1 ± 3.3	6.0 ± 1.9	10.5 ± 1.5	13.3 ± 1.8	21.1 ± 1.9
Scanning (bromphenol blue)	43.8 ± 4.0	5.6 ± 0.8	12.2 ± 2.1	15.6 ± 1.4	22.7 ± 3.0
"Corrected" scan (bromphenol blue)	52.4 ± 5.3	4.1 ± 0.5	10.2 ± 2.3	13.8 ± 1.5	19.7 ± 2.8
"Analytrol" (bromphenol blue)	58.6 ± 7.0	3.4 ± 0.7	8.0 ± 2.7	12.5 ± 3.2	17.6 ± 3.1

*Means and standard deviations expressed as per cent of total protein.

Durrum.[12] Following densitometry the bromphenol blue-stained strip was immersed in ether to remove the oil, transected at 5 mm. intervals, and eluted into 0.01 N NaOH.[12]

The mean values for the separated fractions, expressed as per cent of total protein, are presented in Table I. It will be noted that the albumin concentrations found on densitometry were significantly lower than those obtained by elution. Since the maximum scanned densities of the albumin peaks ranged from 0.34 to 0.58 with bromphenol blue and from 0.53 to 0.77 with Amidoschwarz, the linear range of the densitometer was exceeded in every instance. It appears likely, therefore, that an instrument error was largely responsible for the difference between the scanned and eluted results. However, compensating for the error by converting the scanned densities to their corresponding elution values on the basis of the calibration curve in Fig. 4 did not fully correct for the difference (Table I). Moreover, the discrepancy between the two values in individual samples varied over a much wider range than could be accounted for on the basis of an instrument error alone (Table II). This suggested that other factors, and especially irregularities in the distribution of dye, contributed to the scanning error. Staining errors and losses of albumin due to trailing could not have contributed to the depression of the scanned values, since the same strip was used for both densitometry and elution.

TABLE II. PERCENTAGE DEVIATION OF SCANNED VALUES FROM THEIR CORRESPONDING ELUTION VALUES IN PAPER ELECTROPHORETIC ANALYSES OF 10 NORMAL SERA*

METHOD	ALBUMIN	GLOBULINS			
		α_1	α_2	β	γ
Scanning (Amidoschwarz 10B)	−15 ± 8	+95 ± 94	+23 ± 23	+20 ± 27	+20 ± 23
Scanning (bromphenol blue)	−24 ± 4	+70 ± 33	+42 ± 17	+35 ± 23	+29 ± 19
"Corrected" scan (bromphenol blue)	−11 ± 4	+24 ± 23	+23 ± 13	+19 ± 13	+11 ± 16
"Analytrol" (bromphenol blue)	+1 ± 12	+6 ± 35	−8 ± 25	+8 ± 31	−1 ± 20

*Each value represents the mean of the percentage deviations of the scanned values from their corresponding elution values in 10 paired analyses ± the standard deviation of the mean.

TABLE III. PERCENTAGE DEVIATION OF FILTER PAPER VALUES FROM THEIR CORRESPONDING TISELIUS VALUES IN ELECTROPHORETIC ANALYSES OF 10 NORMAL SERA*

METHOD	ALBUMIN	GLOBULINS			
		α_1	α_2	β	γ
Elution (bromphenol blue)	+1 ± 8	−25 ± 26	+21 ± 14	−21 ± 16	+18 ± 16
Scanning (Amidoschwarz 10B)	−15 ± 6	+37 ± 46	+49 ± 24	−13 ± 11	+40 ± 22
Scanning (bromphenol blue)	−24 ± 7	+23 ± 27	+70 ± 13	+7 ± 16	+50 ± 24
"Corrected" scan (bromphenol blue)	−12 ± 10	−6 ± 23	+40 ± 16	−8 ± 17	+30 ± 22
"Analytrol" (bromphenol blue)	−1 ± 9	−23 ± 20	+10 ± 29	−18 ± 19	+15 ± 17

*Each value represents the means of the percentage deviations of the filter paper values from their corresponding Tiselius values in 10 paired analyses ± the standard deviation of the mean.

The *elution* values for albumin were in reasonably close agreement with those obtained by the Tiselius method (Table I). Higher values might have been anticipated since the dye-binding capacity of albumin is greater than that of globulin.[1, 8, 12] However, trailing, which increases the globulin fractions at the expense of albumin, appeared to compensate for this tendency. In contrast, the *scanned* values were significantly lower than those found on free electrophoresis (Table I), which again pointed to an instrument error. However, as before, the wide range of differences between the two values in individual sera (Table III) indicated that other factors were also operative.

Obviously the decrease in albumin due to scanning errors was accompanied by a proportionate increase in the globulins (Table I). However, the percentage deviations of the individual fractions from their corresponding elution and Tiselius values were larger and more erratic than in the case of albumin (Tables II and III). This was to be expected since scanning errors of the same magnitude produced proportionately larger deviations in fractions of relatively low concentration.

Analytrol Data.—In contrast to the other densitometers tested, the Analytrol responded linearly to increasing concentrations of electrophoresed albumin (Fig. 7) and yielded reasonably accurate results in analyses of serum, suggesting that the compensating cam incorporated in the instrument was effective in reducing scanning errors to a minimum. As indicated in Table I, the *mean* values for the separated fractions of normal serum were in close agreement with those obtained by the elution and Tiselius methods. However, when the results were compared in *individual* samples, significant discrepancies were noted (Tables II and III). These did not appear to be the consequence of erratic instrument behavior, since replicate analyses of a single strip were in close agreement (Table IV). Nor could they be ascribed to variations in the texture of the filter paper strips, since the latter were found to be remarkably uniform in density, even after subjection to the entire staining procedure. However, when multiple strips were streaked with the same sample of serum and subjected to electrophoresis, staining, and scanning under identical conditions, the results showed a significant range of variation, particularly in the case of the globulins

TABLE IV. REPRODUCIBILITY OF THE RESULTS* OBTAINED WITH THE ANALYTROL COMPENSATING RECORDING DENSITOMETER IN FILTER PAPER ELECTROPHORETIC ANALYSES OF SERUM

ANALYSIS	ALBUMIN	GLOBULINS			
		α_1	α_2	β	γ
Single electrophoretic pattern of normal serum scanned 8 times	66.4 ± 0.9 (1.3%)	3.9 ± 0.2 (5.1%)	11.5 ± 0.4 (3.5%)	10.4 ± 0.4 (3.8%)	7.6 ± 0.2 (2.6%)
Replicate analyses (8) of single samples of normal serum:					
A	52.8 ± 3.7 (7.0%)	5.1 ± 0.9 (17.6%)	8.9 ± 1.2 (13.5%)	13.9 ± 1.7 (12.2%)	19.3 ± 2.2 (11.4%)
B	67.7 ± 4.0 (5.9%)	3.6 ± 0.8 (22.2%)	5.9 ± 0.9 (15.3%)	9.2 ± 1.2 (13.0%)	13.6 ± 2.2 (16.2%)

*Means and standard deviations expressed as per cent of total protein. Values in parentheses represent the coefficients of variation ($\frac{\text{standard deviation}}{\text{mean}} \times 100$).

(Table IV). Irregularities in the distribution of dye-stained protein on paper appeared to be the most likely explanation for this observation, and also, in part at least, for the erratic differences between the Analytrol, elution, and Tiselius values observed in individual samples (Tables II and III). No doubt errors peculiar to the elution and Tiselius methods also contributed to these differences.

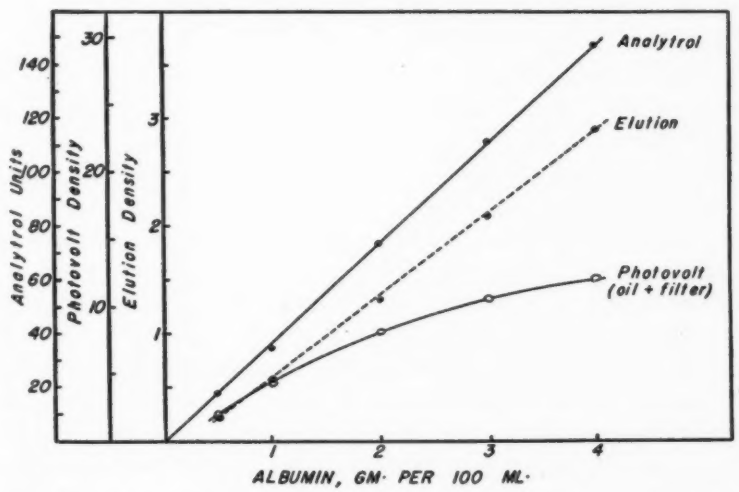

Fig. 7.—Response of the Analytrol compensating recording densitometer to increasing concentrations of albumin in electrophoresed streaks stained with bromphenol blue. The results are compared with those obtained on scanning the same strips in the Photovolt densitometer and following elution.

DISCUSSION

The evidence presented clearly indicates that, within the range of concentrations usually found in filter paper electrophoretic patterns of serum, stained albumin does not obey Beer's law on direct densitometry. As a result, the scanned values for serum are erroneously low in albumin and high in globulin, an artifact which is increased by albumin trailing but which is not nullified by

the greater dye-binding capacity of albumin. In contrast, the densities of the individual globulin fractions in normal serum are low and fall on the relatively linear portion of the density-concentration curve, so that they are little affected by scanning errors. Under pathologic conditions, however, one or more of the globulin peaks may be high, so that deviations from Beer's law become evident. The net effect cannot be predicted, since the scanned values, expressed as per cent of total protein, depend not only on the absolute densities of the individual fractions but also on their relative densities and on the amount of albumin trailing.

From the present study it would appear that the deviations from Beer's law encountered in scanning stained protein are due primarily to an instrument error related to the use of nonmonochromatic light. However, Jencks, Jetton, and Durrum[12] found that even with monochromatic light the Durrum-Gilford densitometer[11] yielded falsely low values for albumin in analyses of serum, an error attributed to irregularities in the distribution of dye on paper. No doubt such irregularities are unavoidable, even with the most meticulous technique, and contribute to scanning errors by lowering the density more markedly at high than at low concentrations. Nevertheless, it is possible that the densities of the albumin peaks studied by Jencks and associates exceeded the linear range of their instrument for bromphenol blue. Although they showed that the response of the instrument to an optical wedge was linear, they did not investigate its response to solutions of dye. This may be of significance since in the present study the instrument responses to an optical wedge and dye solutions differed.

Theoretically, reducing the amount of serum applied to the filter paper strip should eliminate instrument errors by keeping the maximum density of the albumin peak within the relatively narrow linear range of the photometer. However, this lowers the concentration of the globulin fractions to the point where albumin trailing, galvanometer fluctuations, "edge effects," and unavoidable inaccuracies in graphing and planimetry become major sources of error. On actual test it was found that the results obtained with volumes of serum below 0.005 ml. were unreliable. Oiling the paper also reduces density by minimizing light scatter. However, it does not eliminate instrument errors, since it does not lower the density of the albumin peak in normal serum to within the linear range of the densitometer.

Since the dye-binding capacities of the individual fractions of serum protein differ,[1, 8, 12] the question arises whether correction factors are essential in analyzing filter paper electrophoretic patterns. Both in the present study and in others[17] the uncorrected elution values were in reasonably good agreement with those obtained by the moving boundary method, although the ratios between the two values in the individual fractions showed marked variations. This suggests that the errors inherent in the filter paper method tend, in general, to compensate for differences in dye-binding, but that the direction and magnitude of these errors are unpredictable. It would serve no useful purpose, therefore, to introduce a set of correction factors. This opinion is shared by many others.[1, 8, 12, 17]

Quite apart from the uncorrectable errors inherent in the filter paper method related to irregularities in the distribution of dye and differences in dye-binding, there are a number of artifacts which may be introduced during the drying, staining, and fixing procedure. Jencks, Jetton, and Durrum[12] have investigated these in great detail and have proposed a method which is worthy of general adoption.

Of the several methods available for analyzing filter paper serum electrophoretic patterns, direct scanning in a compensating recording densitometer would appear to be the one of choice. The instrument tested in the present study, the Analytrol, appeared to correct for scanning errors, yielded reproducible results, and had the added advantage of speed and simplicity of operation. It did not, however, eliminate some of the errors inherent in the filter paper method.

SUMMARY

Streaks of albumin electrophoresed on paper and stained with bromphenol blue or Amidoschwarz showed a deviation from Beer's law when scanned in a densitometer. This was due primarily to an instrument error related to the use of mixed instead of monochromatic light, although other factors, and especially irregularities in the distribution of dye, contributed to the error. The net result in analyzing normal serum was to increase the relative concentrations of the globulin fractions at the expense of albumin, an effect which differences in dye-binding capacity did not correct. Tests of a recently developed compensating recording densitometer revealed that it reduced scanning errors to a minimum and was capable of analyzing serum electrophoretic patterns with reasonable speed and accuracy. However, it did not eliminate some of the errors inherent in the paper electrophoretic method.

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THE DETERMINATION OF ACID AMINOPOLYSACCHARIDE IN URINE

NICOLA DIFERRANTE, M.D., AND CLAYTON RICH, M.D.
NEW YORK, N. Y.

URINARY acid aminopolysaccharide (APS) has been determined by procedures which may be divided into two main groups: those based on the precipitation of APS by means of such reagents as basic lead acetate[1] and benzidine hydrochloride,[2] and those based on the fact that APS is not dialyzable through cellophane.[3] The material obtained by these methods, after partial purification, has been identified or determined by measuring its anticoagulant activity,[2] its metachromasia[2] or, after acid hydrolysis, its glucuronic acid[4] and hexosamine content.[5]

Recently, the formation of insoluble complexes between acid aminopolysaccharides and long-chain quaternary ammonium salts has been described.[6, 7] This property of quaternary ammonium salts has been used to measure isolated acid aminopolysaccharides and to determine hyaluronidase activity.[8]

This paper presents a method in which a quaternary ammonium salt has been used for precipitation of APS from human urine.

REAGENTS

1. Cetyl trimethyl ammonium bromide, 2.5 per cent solution in water.*
2. Ninety-five per cent ethyl alcohol, saturated with sodium chloride.

METHOD

Twenty-four hour urine collections are preserved by refrigeration. Urine with a specific gravity greater than 1.020 is diluted with an equal volume of distilled water but is not filtered. Duplicate 30 ml. aliquots of urine in 40 ml. conical centrifuge tubes are brought to pH 5 with 2 N HCl with use of indicator paper. To each tube, 1 ml. of cetyl trimethyl ammonium bromide reagent is added and mixed. The tubes are kept overnight at 2° C., then centrifuged at room temperature for 15 minutes at 2,000 g. The supernatant fluid is decanted carefully and the tubes are inverted and allowed to drain 1 minute. The precipitate is washed twice with 30 ml. of ethyl alcohol saturated with NaCl, a glass rod being used to disperse the precipitate before each centrifugation. Each precipitate is dispersed to aid solution after addition of about 1.5 ml. distilled water and 2 drops of 0.1 N NaOH. The solution is transferred quantitatively, with several washings, to a 5 ml. volumetric flask and brought to volume with water. If all of the precipitate does not go into solution in 5 ml. (or if the subsequent optical density is greater than 0.7), the volume is doubled. If any material remains insoluble, its removal by centrifugation will avoid an occasional high blank. On 1 ml. aliquots of this solution, the glucuronic acid content is determined by the carbazole method of Dische.[9]

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RESULTS

The urinary excretion of APS in normal adults and children has been measured with use of the method described. The results, expressed as glucuronic acid, are indicated in Table I.

TABLE I. URINARY EXCRETION OF APS, EXPRESSED AS GLUCURONIC ACID, BY NORMAL ADULTS AND CHILDREN

NUMBER OF PATIENTS	TYPE	RANGE (MG. PER 24 HOURS)	AVERAGE (MG. PER 24 HOURS)
9	Women	2.73- 5.40	3.77 ± 0.240
6	Men	4.40- 7.28	6.00 ± 0.303
4	Girls (7-8 years)	4.80- 8.10	5.80 ± 0.424
25	Boys (7-16 years)	4.10-14.70	7.45 ± 0.314

The differences between the values for women and men and the values for girls and boys are statistically significant. Equally significant are the differences between the values for women and girls and those for men and boys.

Multiple determinations on the same urine showed a standard deviation of ±4.55 per cent. Several factors involved in the precipitation of APS by the cetyl trimethyl ammonium bromide were investigated. To obtain a maximal recovery with any given urine it was found that: (1) the pH of the urine should be between 4 and 6; (2) if the specific gravity is greater than 1.020, or if the urine contains heavy precipitate, it should be diluted with an equal volume of distilled water; (3) the cetyl trimethyl ammonium bromide should be in excess of 0.5 mg. per milliliter of urine and, (4) the mixture of urine and cetyl trimethyl ammonium bromide should be allowed to stand at 2° C. for 15 hours before centrifugation.

The possibility that glucuronic acid, or glucuronic acid containing compounds other than APS, might be precipitated by the cetyl trimethyl ammonium bromide was investigated. The addition of 2 mg. of glucurone, borneol-glucuronide, menthol-glucuronide, phenolphthalein-glucuronide, or pregnanediol-glucuronide to duplicate 40 ml. aliquots of urine did not influence the recovery of APS present. The oral administration of 10 Gm. of glucurone daily to each of 2 patients caused a 70 to 140 per cent increase in the amount of glucuronic acid containing material precipitated from the urine. This increase could be accounted for by the appearance of dialyzable material containing glucuronic acid. After addition of 1.7 to 21.4 mcg. of chondroitinsulfate from cartilage,* or 50.0 mcg. of sodium heparinate or hyaluronate per milliliter of urine to duplicate aliquots, the recovery ranged between 92 and 103 per cent. The recovered heparinate and hyaluronate were clearly distinguished from the APS of urine by their mobilities during paper chromatography, performed according to the method described by Kerby.[4]

The material which is precipitated from the urine by cetyl trimethyl ammonium bromide is strongly metachromatic with toluidine blue. Neither a concentrated aqueous solution of this material nor a similar solution, which

*Chondroitinsulfate from bovine nasal cartilage was kindly made available by D. D. Dziewiatkowski.

had undergone digestion by trypsin, had glucuronic acid containing material which dialyzed through cellophane in 24 hours at 4° C. When the material digested by trypsin was incubated with testicular hyaluronidase for 30 minutes, pH 6, 37° C., there was a 50 per cent depolymerization of the APS present, as demonstrated by a turbidimetric method recently described.[8]

Paper chromatograms[4] and paper electrophoresis (Whatman No. 3 filter paper, acetate buffer pH 4.5, 0.1 μ, 110 volts for 4 hours) were carried out on chondroitinsulfate from cartilage and many samples of APS from urine. When the papers were stained with toluidine blue, the metachromatic material from urine was found to behave similarly to chondroitinsulfate. In addition to this metachromatic material which moved at the solvent front, there was a much smaller amount of metachromatic material at and near the origin. Both of these areas stained faintly with bromphenol blue. In the case of the APS fraction which had been treated with trypsin before the chromatogram was made, only the metachromatic area at the solvent front was present, and this did not stain with bromphenol blue.

DISCUSSION

The method presented, in common with previous methods,[1, 2] relies on the acid character of the APS. That it ordinarily measures a specific substance containing aminopolysaccharide is supported by the following data: (1) the values for normal subjects are low, fairly constant from day to day, and in good agreement with those found by Kerby using a similar method; (2) the values do not change if glucurone, borneol-glucuronide, menthol-glucuronide, phenolphthalein-glucuronide, or pregnanediol-glucuronide are added to urine, but added chondroitinsulfate, sodium heparinate, and hyaluronate are completely recovered; (3) the APS fraction will not dialyze through a cellophane membrane; (4) it is metachromatic and, (5) its behavior during paper chromatography and electrophoresis is similar to that of chondroitinsulfate.

That this method will measure other uronic acid-containing materials is demonstrated by the rise in glucuronic acid values after oral administration of glucurone. Craddock and Kerby,[5] using benzidine as a precipitant, have observed a similar rise and have shown that it is due to dialyzable material and that it is not accompanied by a proportionate increase in hexosamine and, therefore, not due to an increase in aminopolysaccharide excretion.

The presence of material which stains with bromphenol blue before but not after the action of trypsin suggests that the APS precipitated by this method is associated with another substance which is free of glucuronic acid and is probably a protein.

The nature of the APS fraction and its qualitative and quantitative variation in disease states is under study.

SUMMARY

1. A method is presented for the determination of urine aminopolysaccharide by its glucuronic acid content after precipitation with cetyl trimethyl ammonium bromide.

2. The conditions are outlined for maximal yield from urine and for complete recovery of added chondroitinsulfate.

3. The similarity of the APS fraction to chondroitinsulfate is indicated.

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